Regulation of Glutathione S-Transferases During Stress

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I declare that this thesis has been composed by myself and that the work is either my own or that the author is clearly stated.

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ABSTRACT

Elevation of glutathione S-transferases (GST) in tumour cells can be responsible for resistance to a variety of chemotherapeutic agents. It has been hypothesised that GSTs may be induced as part of a stress response, similar to that of the prokaryotic adaptive response. To further this investigation I studied the induction of GSTs in a variety of permanent and transient stress models. A chlorambucil resistant CHO cell line that was known to express increased levels of Alpha-class GST was studied to determine the nature of the increase in protein. Northern and Southern blot analysis revealed a 4-8 fold amplification in the DNA encoding the Alpha-class GSTs with an accompanying increase in mRNA levels. Elevated levels of an Alpha-class GST were noted in oxygen resistant CHO cells. Transient exposure to 98% oxygen also induced the same Alpha-class GST. A heat shocked lung tumour cell line as well as heat selected sublines showed some changes in the levels of Pi-and Mu-class GSTs. A novel putative Mu-class GST subunit has been identified in the nucleus of heat shocked cells.

The nature of the GST level variations at the RNA and DNA levels were studied. These studies do not suggest co-ordinate regulation of the GSTs as part of a general stress response. It does not exclude the possibility of GST π and perhaps the nuclear Mu-class GST are induced as part of a more limited response either to heat or in certain tissues.

Inconsistencies in the data from the preliminary induction experiments led to the investigation of the effect of growth conditions on GST levels. Unexpectedly isoenzymes from three classes of GST were

found to be elevated by increased confluence and a low frequency of feeding. This response was found to be mediated through the culture media. Preliminary analysis suggests the factor responsible has a molecular weight of less than 14 kD but it is not due to an alteration in the pH or redox balance of the media. The regulation of this response was studied and evidence suggests this response is due to a change in protein stability or translational efficiency.

This response of GST to high densities and under feeding may have relevance to drug resistance in solid tumours.

Abbreviations

Ab	antibody
BCNU	1,3-bis-(2-chloroethyl)-1-nitrosourea
BSO	Buthionine Sulphoxamine
CDNB	1-chloro-2,4-dinitrobenzene
CHO	Chinese hamster ovary
CuOOH	cumene hydroperoxide
DTT	dithiothreitol
EA	ethacrynic acid
EtOH	ethanol
γGT	γ-glutamyl transpeptidase
GSH	reduced glutathione
GSSG	oxidised glutathione
GST	glutathione S-transferase
hsp	heat shock protein
IHC	immunohistochemistry
NSCLC	non small cell lung carcinoma
PBS	phosphate buffered saline
PCR	polymerase chain reaction
P-gp	P-glycoprotein
SCLC	small cell lung carcinoma
SOD	superoxide dismutase

Table of Contents

I. Introduction 2
I.1. Drug metabolism in mammals 3
I.1.1. Glutathione and its metabolism 4
I.1.2. Glutathione related enzymes 5
I.2. Glutathione S-transferases 6
I.2.1. Nomenclature 6
I.2.2. GST subunits 7
I.2.2a. Alpha class GST 13
I.2.2b. Mu class GST 14
I.2.2c. Pi class GST 15
I.2.2d. Theta class GST 16
I.2.2e. Microsomal GST 16
I.2.3. Regulation of GST subunits 16
I.2.4. The reactions of GST in the cell 17
I.3. Stress in the environment 20
I.3.1. The Stress Response 21
I.3.2. Stress Proteins 21
I.3.3. Regulation of the response 23
I.4. The role of GSH in stress 24
I.5. Agents used in chemotherapy 26
I.6. Cross resistance to several drugs 27
I.7. Adaptive response in preneoplasia 28
I.8. Aims 31
II. Materials and Methods 33
II.1. Materials 33
II.1.1. Cytotoxic drugs 33
II.1.2. Cell lines 34
II.1.3. Antibodies 34

II.1.4. GST standards for Western analysis 36
II.1.5. DNA probes 36
II.2. Methods 37
II.2.1. *In vitro* cell culture techniques 37
II.2.1a. Culture conditions 37
II.2.1b. Cell line storage 37
II.2.2. Cytotoxicity assays 38
II.2.2a. MTT assay 38
II.2.2b. Establishment of the number of NCI H322 cells to use for MTT assay 39
II.2.3. Protein analysis 40
II.2.3a. Immunohistochemistry 40
II.2.3b. Protein preparations 40
II.2.3c. Protein estimation 41
II.2.3d. Western blot analysis 42
II.2.4. Nucleic acid analysis 43
II.2.4a. Plasmid DNA preparations 43
II.2.4b. Genomic DNA preparations 44
II.2.4c. RNA preparations 45
II.2.4d. Southern blot analysis 45
II.2.4e. Northern blot analysis 47
II.2.4f. PCR 48
III. Preliminary analysis of GST in various stress models 50
III.1. Changes in protein expression following NCI H322 heat shock. 54
III.2. Further investigation of expression of GST in heat shocked NCI H322 cells 63
III.3. Summary and discussion 67
IV. GST expression in chlorambucil and oxygen resistant CHO cells 72
IV.1. Oxygen resistance and transient oxidative stress 73
IV.2. Chlorambucil resistance 76

IV.3. Discussion 86
IV.3.1. Chlorambucil resistance 86
IV.3.2. Oxygen stress and resistance 87
IV.3.3. Link between chlorambucil and oxidative stress 89
V. GST's are regulated by a conditioned media response in a lung tumour derived cell line NCI-H322 91
V.0.1. Role of GST in the lung 91
V.1. Western Blot analysis of GST content of NCI H322 cells 93
V.2. Effect of time in culture on Alpha and Pi class GSTs 99
V.3. Effect of culture conditions on the GST content of NCI H322 cells 99
V.4. Induction of GST is mediated by the culture media 108
V.5. Regulation of GST at the molecular level 114
V.6. Discussion 119
V.6.1. GST subunits in NCI H322 cells 122
V.6.2. Identification of the cytosolic Mu-class GST 123
V.6.3. Identification of the Alpha class subunit(s) 125
V.6.4. Regulation of the conditioning response 126
V.6.5. Heterogeneity of the conditioned response 126
VI. Analysis of GST expression during heat shock 129
VI.1. GST expression following 42°C heat shock 129
VI.1.1. Induction of a nuclear Mu-class GST during heat shock 130
VI.1.2. Pi class GST cross reactivity in the nucleus of heat shocked cells 136
VI.1.3. GST expression following 42°C heat shock, allowing for induction of GST by the conditioning response 144
VI.1.4. Lack of transcription of Pi class GST in heat shocked cells 144
VI.2. 45°C heat shock of NCI H322 cells 144

VI.3. Effect of heat shock on the drug sensitivity of NCI H322 cells 149
VI.4. Discussion 156
VI.4.1. Pi class GST as a heat shock protein 156
VI.4.2. A novel nuclear Mu class GST 158
VI.4.3. Are the GST subunits detected in the nucleus contaminants from the cytoplasm? 159
VI.4.4. The role of GST in the nucleus 161
VII. Development and analysis of heat selected cell sublines 164
VII.1. Development of heat selected cell lines 165
VII.2. Immunohistochemical analysis of the cell sublines for GST content 168
VII.2.1. Alpha class glutathione S-transferase 172
VII.2.2. Mu class glutathione S-transferase 172
VII.2.3. Pi class glutathione S-transferase 173
VII.3; Western blot characterisation of the GST content of three heat selected sublines 173
VII.3.1; Alpha class glutathione S-transferase 177
VII.3.2. Mu class glutathione S-transferase 177
VII.3.3. Pi class glutathione S-transferase 180
VII.4. Northern analysis of Pi class GST mRNA levels 181
VII.5. Southern Analysis of heat selected sublines 181
VII.6. Drug sensitivities of the heat selected sublines 181
VII.7. Heat sensitivity of the sublines 194
VII.8. Discussion 201
VIII. Summary and concluding remarks 206
IX. References 211

List of Figures and Tables

Table 1; Glutathione S-transferase subunits of human. 8

Table 2 Glutathione S-transferase subunits of rat. 9

Table 3; Comparison of GST subunit nucleotide sequences 11

Table 4; The antisera used to detect GST subunits 35

Table 5; Results of immunohistochemical analysis. 52

Figure 1; Light micrographs of heat shocked NCI H322 cells stained for Alpha, Mu and Pi class glutathione S-transferases and topoisomerase II described in Table 5. 56

Figure 2; Confocal microscopy of Alpha, Mu and Pi class GST and topoisomerase II immunohistochemistry staining in heat shocked NCI H322 cells. 58

Figure 3; Densitometry of immunohistochemistry results for heat shocked NCI H322 cells. 61

Figure 4; Glutathione S-transferase levels in heat shocked NCI H322 cells up to day 10 recovery. 64

Figure 5; Heterogeneity of staining for GST in confluent NCI H322 cells. 68

Figure 6; GST protein expression in oxygen sensitive and oxygen resistant CHO cell lines. 74

Figure 7; Alpha class GST mRNA in chlorambucil and oxygen resistant and oxygen treated CHO cell lines. 77

Figure 8; Amplification of DNA encoding Alpha class GST in a chlorambucil resistant cell line. 80

Figure 9; Southern analysis of gGT in a chlorambucil resistant cell line. 82

Figure 10; Estimation of fold amplification of Alpha class GST in a chlorambucil resistant cell line. 84

Figure 11; Expression of GST protein in control NCI H322 cells 94

Figure 12; Southern blot analysis of GST-M1 in NCI H322 cells 97

Figure 13; Induction of Pi and Alpha class GSTs during culture of NCI H322 cells. 100

Table 6; Protocol for testing effect of feeding and density on GST expression (figure 15). 102

Figure 14; NCI H322 cells before harvesting for figure 15. 104

Figure 15; Effect of density and feeding of NCI H322 cells on expression of GSTs. 106

Figure 16; Effect of conditioning media and varying pH on expression of GST 109

Figure 17; Effect of dialysis and proportion of conditioning and fresh media on the ability to alter GST expression. 112

Figure 18; Effect of altering the glutathione and redox state of the media on the NCI H322 conditioning response.....115
Figure 19; Effect of NCI H322 conditioned media on expression of Alpha class GST from a heterologous promoter. 117
Figure 20; Levels of Pi class GST mRNA in conditioned NCI H322 cells.....120
Figure 21; Morphology of heat shocked NCI H322 cells.....131
Figure 22; Western blot analysis of Mu class GST staining in the nucleus of cells heat shocked at 42°C.....134
Figure 23; Western blot analysis of Pi class GST in cells heat shocked at 42°C.....137
Figure 24; Depletion of Pi class GST antisera. 139
Figure 25; Use of disruption methods on GST-P1 immunoreactive protein.....142
Figure 26; Expression of GST in cytosol of 42°C heat shocked NCI H322 cells. 145
Figure 27; Northern analysis of 42°C heat shocked cells.....147
Figure 28; Expression of GST in 45°C heat shocked NCI H322 cells.....150
Figure 29; Effect of heat shock on NCI H322 cells' drug sensitivity.....152
Figure 30; Effect of heat shock and varying recovery period on the drug sensitivity of NCI H322 cells. 154
Figure 31; Protocol for the selection of heat resistant NCI H322 cells.....166
Figure 32; Cell lineage of heat selected NCI H322 sublines. 169
Table 7; Expression of GSTs in heat selected cells measured by immunohistochemistry. 170
Figure 33; Heat selected NCI H322 sublines. 175
Figure 34; Expression of GSTs in heat selected NCI H322 cells measured by Western blot. 178
Figure 35; Northern blot analysis of the heat selected cell sublines. 182
Figure 36; Southern blot analysis of the heat selected cell sublines 184
Figure 37; Comparison of the growth characteristics of heat selected NCI H322 cells.....188
Figure 38; Estimation of drug concentrations to use in the heat treated cell subline cytotoxicity assays.....190
Figure 39; Difference between measuring fold resistance and cell survival.....192
Figure 40; Estimation of drug sensitivities of the heat selected cell sublines using the MTT assay.....195
Table 8; Drug sensitivities of the heat selected sublines 198
Table 9; Heat sensitivity of the heat selected sublines as measured by MTT assay. 200
Table 10; Summary of immunological and cytotoxicity data.....202

CHAPTER I

Introduction

I. INTRODUCTION

One of the major reasons for the failure of chemotherapy is the development of drug resistance. In cancer treatment, drug resistance is manifest either as intrinsic or acquired drug resistance. Intrinsic drug resistance occurs when a tumour, such as colon or certain types of skin cancer, responds poorly to chemotherapy even at the initial treatment. Acquired drug resistance occurs when a tumour, such as small cell lung cancer or breast cancer, which is initially sensitive to treatment becomes resistant to further treatment. It is thought to arise as a result of changes in a single cell of the tumour. This leads to the outgrowth of a resistant cell population that has a selective advantage over the original primary tumour.

A cell may become resistant to chemotherapeutic drugs through a variety of mechanisms. For example the cell may have mechanisms that reduce the effective concentration of the reactive compound. This may be achieved by several means. At the cell membrane, the drug may be prevented from entering (decreased influx) or once having entered the cell may be expelled at a higher rate (increase in drug efflux). Alternatively the metabolism of the drug may be altered. A drug that requires cellular activation may be metabolised to its active form more slowly (generally phase I drug metabolising enzymes) or an active compound may be detoxified more rapidly (phase II drug metabolising enzymes). The cell may also be able to protect a critical target site from damage or the cell may repair or replace cell components, after the damage has occurred.

Resistance to chemotherapeutic agents is a major clinical problem in the treatment of most cancers. The primary aims of this thesis are to

investigate mechanisms behind the emergence of drug resistant tumours during chemotherapy. Of particular interest to our laboratory is the role played by a family of phase II drug metabolising enzymes, the glutathione S-transferases (GST). The investigation described here focuses on the proposal that the glutathione S-transferases are induced by various stresses as part of an adaptive response similar to that seen with heat shock. In this chapter I will discuss the glutathione S-transferases, structure, function, their involvement in drug resistance and the possible relationship between glutathione S-transferases, stress response and drug resistance.

I.1. Drug metabolism in mammals

The drug metabolism system of the higher eukaryotes is very extensive; not only does it have to cope with a wide variety of toxins ingested every day; it is also involved in metabolising many endogenous compounds. The major drug detoxification system in mammals is composed of the phase I and phase II drug metabolism enzymes. The cytochrome P450 enzymes are the major phase I enzymes. These tend to be involved in converting various compounds into suitable substrates for the phase II enzymes. This process also tends to make the compounds more reactive and as a consequence often more toxic to the cell. The phase II enzymes include the glutathione related enzymes and the UDP-glucuronyl transferases. These are involved in detoxifying reactive compounds in preparation for excretion from the body.

I.1.1. Glutathione and its metabolism

One of the most important multifunctional molecules in the aerobic cell is glutathione (Meister and Anderson, 1983; Wolf *et al*, 1987). Glutathione is a tripeptide consisting of glutamine, cysteine and glycine and is the most abundant intracellular thiol present in millimolar concentrations. The reduced (GSH) and the oxidised (GSSG) forms, exist in equilibrium in the cell. Severe depletion of GSH affects the thiol disulphide state of proteins and the redox balance of the cell (reviewed in Uhlig and Wendel, 1992). In its reduced state it is a cosubstrate for the glutathione S-transferases, for the detoxification of electrophilic compounds and the reduction of hydroperoxides. Glutathione is essential for both the synthesis and the degradation of proteins and for the synthesis of DNA precursors (Kosower and Kosower, 1979; Holmgren, 1990). The diverse role of glutathione includes the metabolism of a variety of compounds such as leukotrienes, prostaglandins and oestrogens (Ujihara *et al*, 1988; Soderstrom *et al*, 1985; Yoshimoto *et al*, 1988). In addition glutathione is nucleophilic and can be conjugated to a variety of electrophiles, which results in detoxification and increased polarity hence facilitating the excretion of the compound. A wide variety of foreign compounds can be detoxified by this method, including a number of cytotoxic drugs commonly used in chemotherapy. In combination with the inorganic and organic peroxidase activity of glutathione peroxidase, the organic glutathione peroxidase activity of some of the glutathione S-transferases is important in protecting the cell from oxidative damage caused both by side products of oxidative phosphorylation and from a variety of external sources.

I.1.2. Glutathione related enzymes

Glutathione synthetase and gamma glutamyl synthase are both involved in the manufacture of GSH. Glutathione reductase is involved in converting GSSG to GSH, in readiness for its use in the cell as a reducing agent either enzymatically or without catalysis. Glutathione peroxidase will reduce both organic and inorganic hydroperoxides and is known to be selenium dependent having a selenocysteine moiety in its active centre. Eukaryotes also have a selenium independent glutathione peroxidase activity that is associated with the enzyme glutathione S-transferase. The glutathione S-transferases also have a variety of other activities including glutathione conjugation and ligand binding (as described later on p17).

Another enzyme that is important in glutathione metabolism is γ-glutamyl transpeptidase (γGT). This enzyme is found induced in many models for preneoplasia and drug priming (Roomi *et al*, 1985; Carmichael *et al*, 1986). The exact role of γGT in both preneoplasia and in the normal cell is not very clear (reviewed by Hanigan and Pitot, 1985). As well as being the sole enzyme known to be capable of degrading glutathione, γGT is the first step in the conversion of glutathione conjugates to mercapturic acids and N-acetylated products (Inoue, 1985). The primary location is in the kidney, although γGT is also found in the pancreas, bile ducts and the bile canalicula (Tate and Meister, 1976). γGT is a plasma membrane protein and is found with its active site orientated towards the exterior of the cell (Meredith and Williams, 1986). It is thought that both oxidised and reduced glutathione and glutathione conjugates, which are excreted from different cell types, may be substrates for this enzyme (Meister 1984; Moss *et al*, 1984). The glutathione is broken down by γGT into its constituents Glu and Cys-Gly

which may then be taken up by the cell. Curthoys and Hughey (1978) believe the major role to be this degradation of GSH and its derivatives and not in the uptake of amino acids. Meister (1973), on the other hand proposes that the principle role is in the uptake of amino acids. This role may confer an advantage to cells with higher levels of the enzyme such as in preneoplastic lesions and stressed cells, enabling replacement of cellular levels of reduced glutathione faster due to their increased concentrations of the constituents (Hanigan and Pitot, 1985).

I.2. Glutathione S-transferases

The group of enzymes that catalyse the conjugation reactions with GSH are the glutathione S-transferases (EC 2.5.1.18). Glutathione S-transferases are found in a wide variety of plants, animals and many prokaryotes (reviewed by Mannervik, 1985; Mannervik and Danielson, 1988; Sies and Ketterer, 1988; Hayes *et al*, 1990b). In mammals there are a large number of isoenzymes that fall into several classes. There is one microsomal GST that consists of a trimer of a 17K subunit and four cytosolic GST classes, Alpha, Mu, Pi and Theta (Mannervik *et al*, 1985; Meyer *et al*, 1991). The cytosolic isoenzymes are composed of heterodimers or homodimers of subunits within each class; heterodimers between classes do not occur. Not all possible heterodimers have been described so it is likely that only some subunits can heterodimerise within their class.

I.2.1. Nomenclature

Where possible, I refer to the human GST subunits and isoenzymes by the most recent and least confusing nomenclature (Mannervik *et al,*

1992). Historically, the three cytosolic GST classes were named after the pI's of the human proteins, being named the basic, neutral and acidic classes. As more GST's were discovered it became clear, especially in species other than humans, that, although the isoelectric points do broadly classify the proteins, there is much overlap and it was better to distinguish the classes by their structure and function. The basic, neutral and acidic classes are now known as the Alpha, Mu and Pi classes, after the major human hepatic forms in each family (Mannervik *et al*, 1985). The subunits within these classes are known by a letter representing the class and a number within the class to represent the individual subunit. The dimeric isoenzymes are represented by the class letter and the two numbers representing their subunit constituents; e.g. A1-A2. Allelic forms are differentiated by lower case letters; e.g. M1a or M1b. Subunits have only been included in the nomenclature once fully characterised hence some subunits are referred to by their laboratory nomenclature. Where known, I refer to the human subunits and isozymes by this most recent nomenclature (Mannervik *et al*, 1992). This nomenclature has not yet been extended to the rat and mouse subunits. As there is sometimes confusion over the identity of some of the rat subunits, these are referred to by the nomenclature used in the individual papers. The human and rat subunits and their old and new nomenclatures are shown in tables 1 and 2.

I.2.2. GST subunits

The individual subunits have a molecular weight of between 22 000-27 000. Each enzyme has a different spectrum of substrates. The subunits are distinguished on the basis of mobility on SDS-PAGE, isoelectric point, substrate specificity, inhibitor kinetics, immunology and

Table 1: Glutathione S-transferase subunits of human.

[1] Class	new [2] nomenclature	old nomenclatures		
Alpha	A1	GST 2-type-1	B_1	Ha-1
	A2	GST 2-type-2	B_2	Ha-2
		GST 9.9[3]		
Mu	M1a	GST 1-type-1	μ	Hb
	M1b	GST 1-type-2	ψ	Hc
	M2	GST 4 [4]	muscle	
	M3	GST 5 [5]	brain	
	M4	GST 5.2 [6]		
	M5	GST 6 [7]		
	P1	GST 3	π [8]	
Theta	T1	θ [9]		
	T2?			
		ζ? [10]		
Microsomal	[11]			

1 Table adapted from Mannervik and Danielson, 1988
2 nomenclature based on Mannervik *et al* (1992)
3 Del Boccio *et al* (1992)
4 Laisney *et al*, 1984; Suzuki *et al*, 1987
5 Laisney *et al*, 1984; Suzuki *et al*, 1987
6 Singh *et al*, 1988
7 Suzuki *et al*, 1991
8 Marcus *et al*, 1978; Guthenberg *et al*, 1979; Koskelo *et al*, 1981
9 Meyer *et al*, 1991
10 Singh *et al*, 1988
11 Morgenstern and DePierre, 1983

Table 2 Glutathione S-transferase subunits of rat.

12	13		
Alpha	Yc	2	
	Ya_1	1	
	Ya_2	8	
		10	
	Yk		
Mu	Yb_1	3	
	Yb_2	4	
	Yb_3, Yn_1	6	
	Yn_2	9	
		11	
	Yb_4		
	Yo		
	Yl		
Pi	Yp, Yf	7	
Theta		12	
		5	GST E
		Yrs	

12 Table adapted from Mannervik and Danielson, 1988

13 nomenclature based on mobility on SDS-PAGE (Hayes, 1986; Hayes and Mantle, 1986a, 1986b)

finally sequence homology (Jakoby, 1978; Chassead, 1979; Mannervik and Danielson, 1988; Bora, 1986; Boyer, 1989; Hayes and Mantle, 1986; Hayes *et al*, 1987a, 1987b). There are a variety of problems associated with these techniques making characterisation of GST subunits complicated, such as overlapping substrate specificity's and the large number of closely related subunits causing immunological cross reactivity. The subunits can also be post translationally modified causing variation in M_r on SDS-PAGE that can make identification by size difficult. Modifications identified so far include calmodulin mediated methylation, acetylation of the initiating methionine and protein kinase C dependant phosphorylation.

Characterisation of the different subunits within the separate families and their expression in different tissues is not at all complete. There is approximately 30% similarity between the different GST classes and 70% similarity within the classes (see table 3). There are many different subunits and these vary among different species (Mannervik and Danielson, 1988). This is a result of continuous evolutionary diversification within the species. The microsomal GST subunit shows no significant homology to the cytosolic forms. The similarity between the different subunits and GST classes are shown in table 3. The relationship of corresponding subunits between species is also not clear. In some families such as the Pi and the microsomal classes, the mammalian species seem to have definitive orthologous subunits. With the Alpha and Mu classes however the classes are much more complicated with some subunits in one species having no obvious counterpart in another. For instance, in rat the two major hepatic Alpha class subunits Ya and Yc are approximately 70% identical, whereas in human the major Alpha class subunits, A1 and A2, which are 95% homologous, are not orthologous to either Ya or Yc but can be considered to

Table 3: Comparison of GST subunit nucleotide sequences

The complete coding region sequence from GST subunits in Genbank-EMBL database were compared and a table of percentage similarity compiled using the Sequence Analysis Software Package Version 7.2 from the Genetics Computer Group (1991).

				Theta			Alpha						Pi			Mu											mic
			reference	rat Yrs	mus 5.7	rat 8	mus Yc	rat Yc	hum A1	hum A2	rat Ya	mus Ya	hum P1	mus Yf	rat Yf	hum M1	hum testis	hum M2	rat Yb_3	ham Yb	rat Yb_2	mus Yb_2	mus Yb_1	rat Yb_1	mus GT9.3	hum M3	hum
Theta	rat	Yrs	Ogura *et al* (1991)	1.00	0.33	0.31	0.33	0.33	0.35	0.35	0.34	0.34	0.34	0.32	0.33	0.32	0.34	0.31	0.33	0.31	0.32	0.32	0.32	0.32	0.33	0.31	0.28
	mouse	5.7	Zimniak *et al* (1992)		1.00	0.90	0.66	0.64	0.66	0.65	0.65	0.66	0.44	0.44	0.43	0.39	0.39	0.39	0.39	0.37	0.37	0.38	0.38	0.39	0.39	0.37	0.30
	rat	8, Ya_2	Stenberg *et al* (1992)			1.00	0.65	0.64	0.66	0.65	0.66	0.66	0.43	0.41	0.41	0.39	0.39	0.38	0.38	0.36	0.36	0.38	0.39	0.38	0.38	0.36	0.27
	mouse	Yc	Hayes *et al* (1992), Buetler and Eaton (1992)				1.00	0.89	0.77	0.77	0.73	0.75	0.42	0.43	0.43	0.38	0.38	0.38	0.37	0.37	0.36	0.37	0.37	0.37	0.37	0.38	0.28
Alpha	rat	2, Yc	Tu *et al* (1984), Telakowski-Hopkins *et al* (1985)					1.00	0.80	0.79	0.75	0.76	0.41	0.43	0.43	0.38	0.39	0.38	0.37	0.37	0.36	0.38	0.38	0.38	0.38	0.37	0.26
	human	A1	Tu and Quian (1986)						1.00	0.96	0.80	0.81	0.43	0.43	0.44	0.38	0.37	0.37	0.37	0.35	0.36	0.36	0.38	0.38	0.38	0.39	0.26
	human	A2	Rhoads *et al* (1987)							1.00	0.79	0.80	0.43	0.44	0.44	0.38	0.38	0.38	0.37	0.36	0.35	0.36	0.38	0.38	0.37	0.40	0.26
	rat	1, Ya_1	Kalinyak and Taylor (1982), Lai *et al* (1984)								1.00	0.94	0.45	0.45	0.45	0.39	0.39	0.39	0.39	0.39	0.38	0.40	0.41	0.40	0.41	0.39	0.25
	mouse	GT41	Pearson *et al* (1988)									1.00	0.44	0.44	0.44	0.40	0.39	0.40	0.39	0.39	0.38	0.39	0.40	0.39	0.40	0.39	0.25
	human	P1	Kano *et al* (1987)										1.00	0.83	0.83	0.45	0.46	0.47	0.47	0.47	0.44	0.46	0.47	0.47	0.45	0.46	0.28
Pi	mouse	Yf	Hatayama *et al* (1990)											1.00	0.93	0.46	0.47	0.47	0.47	0.47	0.44	0.47	0.47	0.47	0.45	0.45	0.28
	rat	7, Yp	Muramatsu (1985)												1.00	0.46	0.46	0.46	0.46	0.46	0.43	0.46	0.46	0.46	0.43	0.45	0.28
	human	M1	Seigegard *et al* (1988), DeJong *et al* (1988b)													1.00	0.90	0.90	0.84	0.82	0.83	0.86	0.83	0.83	0.81	0.74	0.28
	human	testis	Ross and Board (1992) unpublished														1.00	0.86	0.81	0.83	0.71	0.84	0.80	0.79	0.76	0.74	0.26
	human	M2	Vorachek *et al* (1991)															1.00	0.86	0.80	0.80	0.83	0.81	0.82	0.79	0.72	0.28
	rat	6, Yb_3	Abramovitz and Listowsky (1987)																1.00	0.83	0.83	0.84	0.83	0.84	0.82	0.70	0.29
	hams.	gstS6	Norris *et al* (1991)																	1.00	0.88	0.90	0.83	0.83	0.81	0.71	0.29
Mu	rat	4, Yb_2	Lai and Tu (1986), Ding *et al* (1986)																		1.00	0.94	0.83	0.84	0.84	0.70	0.30
	mouse	Yb_2	Townsend *et al* (1989)																			1.00	0.86	0.86	0.85	0.73	0.29
	mouse	Yb_1	Pearson *et al* (1988)																				1.00	0.94	0.89	0.71	0.29
	rat	3, Yb_1	Ding *et al* (1985), Chang *et al* (1987b)																					1.00	0.87	0.70	0.28
	mouse	GT9.3	Pearson *et al* (1983, 1988)																						1.00	0.70	0.29
	human	M3	Campbell *et al* (1990)																							1.00	0.30
Microsomal	human		DeJong *et al* (1988a)																								1.00

have diverged approximately equally from both. This is probably the result of a recent duplication. There is also other evidence of recent evolution in mammalian GST classes with evidence for gene conversion within the Mu class GSTs in rat and human (Lai *et al*, 1988; Taylor *et al*, 1991).

I.2.2a. Alpha class GST

In human adult liver there appear to be two major members of the Alpha class, A1 and A2, that have similar substrate activities. These two subunits share 95% identity at the nucleotide level over the coding region but are encoded by separate genes and are thought to have resulted from a relatively recent gene duplication (Rhoads *et al*, 1987; Hayes *et al*, 1989). Two cDNA's have been cloned corresponding to the two subunits (Tu and Qian, 1986; Rhoads *et al*, 1987; Board and Webb, 1987). Both, the GST-A1 and A2 genes map to chromosome 6p12 (Board and Webb, 1987). Another member of this class with a pI of 9.9 has been described by Del Boccio et al (1987) and is found in human skin. This subunit shows homology to rat subunit 2 (Yc). In rodents the two major members of the Alpha class in liver, Ya and Yc are both equally divergent from the human A1 and A2 proteins.

This class of enzymes is thought to be responsible for the selenium independent glutathione peroxidase activity. This activity is not high in comparison to the activity of glutathione peroxidase nor can they use inorganic hydroperoxide as a substrate but they may still play an important role due to their high concentration within cells. The Alpha class isoenzymes are also responsible for the covalent binding activity, hence their original name of ligandin for the human subunits.

I.2.2b. Mu class GST

The Mu class enzymes are becoming the largest of the GST classes so far. There are three isoenzymes that have been isolated from human livers (Board, 1981b). These are composed of the heterodimer and homodimers of the M1a (μ) and M1b (ψ) subunits. The M1b subunit is only found in around 6% of the population. M1b shows a difference of only 1 amino acid residue from M1a (Seidegard *et al*, 1988; DeJong *et al*, 1988b). The major hepatic Mu class GST-M1 (μ), is polymorphic in the human population with approximately 50% having no detectable protein (Warholm *et al*, 1980; Seidegard and Pero, 1985), resulting from a homozygous null genotype. This genotype, initially detected as a lack of activity for *trans*-stilbene oxide, has been associated with an increased risk of developing lung cancer (Seidegard *et al*, 1986; Seidegard *et al*, 1990). Conflicting data from a PCR based assay however showed no increased susceptibility associated with the GST-M1*0 genotype (Zhong *et al*, 1991).

There are at least two other subunits in this class in human; both are homologous with GST-M1. One form, GST-M2 [GST 4] has been described in human muscle and the other in the brain and testis, GST-M3 [GST-5] (Laisney *et al*, 1984; Suzuki *et al*, 1987). Singh *et al* (1988) has described a further GST in muscle with an N terminal homologous to GST-M1 (GST 5.2). The M1 locus maps to chromosome 1p13, whereas the M2 and M3 genes are located on chromosome 3 (DeJong *et al*, 1988b; Islam *et al*, 1989). Southern blot analysis suggests there may be at least 6 Mu class GSTs in human (DeJong and Tu, 1990). In the rat there are at least five Mu class subunits identified as well as at least two other putative subunits belonging to this class.

I.2.2c. Pi class GST

The major Pi class isoenzyme is the most highly conserved of the cytosolic GSTs. The Pi class is a small gene family with only one member in most species studied so far. Human GST-P1 has been purified from placenta, lung and erythrocytes (Guthenberg *et al*, 1979; Koskelo *et al*, 1981; Marcus *et al*, 1978). Pi class GST is also found in bladder, breast, kidney, platelets, thyroid, heart and spleen (Koskelo, 1983; Sherman *et al*, 1983; Mannervik, 1985; DiIlio *et al*, 1986; Hussey *et al*, 1986; Pemble *et al*, 1987; Singh *et al*, 1987; Tateola *et al*, 1987). It is however only found at low levels in the liver. GST-P1 has been cloned by Kano *et al* (1987) and mapped to chromosome 11q13 (Moscow *et al*, 1988; Board *et al*, 1989). Singh *et al* (1988) has described 2 isoenzymes in skeletal muscle with differing isoelectric points. It is not clear whether these represent different enzymes, alleles or a post-translational modification. Interestingly, two fatty acid ethyl ester synthases were isolated which had CDNB activity and are homologous to GST-P1 (Bora *et al*, 1989a; Bora *et al*, 1989b). Their relationship to P1 is not clear as two groups have since shown separate segregation of fatty acid ethyl ester synthase activity and CDNB activities during protein purification (Suzuki *et al*, 1990; Sharma *et al*, 1991).

I.2.2d. Theta class GST

An isoenzyme isolated from the rat termed GST-E has recently been discovered to be composed of two separate subunits, GST 5 and 12 belonging to a separate class of GST (Meyer *et al*, 1984). A related human GST has also been described by Meyer (1991) as being composed of two separate subunits.

I.2.2e. Microsomal GST

The microsomal GST isoenzyme is quite distinct from the cytosolic GST isoenzymes in size, structure and its location in the microsomal membranes (Morgenstern and DePierre, 1983). It is a trimer composed of a 17 kD subunit that shares no amino acid homology with the cytosolic subunits (DeJong *et al*, 1989; McLellan *et al*, 1989). It is active against cumene hydroperoxide and is activated by N-ethylmaleimide and thought to have a role in prevention of lipid peroxidation (Morgenstern *et al*, 1979).

I.2.3. Regulation of GST subunits

In rat the Alpha and Mu class GST subunits have been shown to be induced by a variety of antioxidants, barbiturates, epoxides and aromatic hydrocarbons (Igarashi *et al*, 1987; Talalay *et al*, 1988; Listowsky *et al*, 1988). The induction of rat Ya in the liver by aromatic hydrocarbons has been shown to be a transcriptional induction dependent on a functional dioxin (Ah) receptor (Ding and Pickett, 1985; Telakowski-Hopkins *et al*, 1988). Induction by antioxidants does not require the Ah receptor and has been shown to be mediated through the antioxidant responsive element (Rushmore *et al*, 1990; Rushmore *et al*, 1991).

The Pi class GST is found induced in a variety of preneoplasia cell models of the liver. In rat GST 7-7 is found expressed 5-7 days after initiation with a carcinogen (Cameron, 1988). Due to this and it's normally low level in the liver, it is the Pi class GST that has been most associated with use as a marker for early detection of preneoplasia or neoplasia (Satoh *et al*, 1985). GST-P1 has been shown to have a TPA responsive element and

its mRNA levels are induced by c-Ha-*ras* and N-*ras* (Power *et al*, 1987; Li *et al*, 1988) but not *fos* or *jun* (Morrow *et al*, 1990). GSTs are also known to be substrates for post translational modifications, methylation, acetylation and phosphorylation. The role of these however is not known but some may affect the activity of the isoenzymes (Tanaguchi and Pyerin, 1989; Johnson *et al*, 1990; Kuzmich, 1991).

I.2.4. The reactions of GST in the cell

The reactions catalysed by the glutathione S-transferases are varied. The GSTs are capable of reacting with a variety of electrophilic compounds, including many toxins and carcinogens (Chasseaud, 1979). This conjugation of glutathione to an electrophilic substrate can be the start of a detoxification pathway leading to the formation of mercapturic acids. Many xenobiotics are excreted in this form. This substrate may be either an endogenous compound such as leukotriene A_4, or an exogenous compound such as cyclophosphamide (Bach *et al*, 1984; Soderstrom *et al*, 1985; Colvin and Hilton, 1987).

The xenobiotic substrates which GSTs have been demonstrated to have a role in detoxifying, include the aromatic hydrocarbons, including metabolites of benzo(a)pyrene and chysene (Jernstrom *et al*, 1985; Robertson *et al*, 1986; Hiratsuka *et al*, 1990). GSTs have also been shown to prevent the formation of DNA adducts by benzanthracene metabolites (Okuda *et al*, 1986; Watabe *et al*, 1987). Several alkylating agents have been shown to be substrates for GST including the nitrogen mustards, melphalan, chlorambucil and cyclophosphamide and the nitrosourea, BCNU (Dulik and Fenselau, 1987; Colvin and Hilton, 1988; Smith *et al*, 1989).

A selenium independent glutathione peroxidase activity also exists and is associated primarily with the Alpha class GST (Jensson *et al*, 1986; Awasthi *et al*, 1980). The substrates of this activity are associated with damage caused by oxidative stress; fatty acid hydroperoxides, hydroxyalkenals, cholesterol epoxide, thymine hydroperoxides and DNA peroxidised by ionising radiation (Meyer and Ketterer, 1982; Alin *et al*, 1985; Jensson *et al*, 1986; Danielson *et al*, 1987; Ketterer *et al*, 1987; Tan *et al*, 1988). The Alpha class GSTs have also been associated with an oxosteriod isomerase activity and are involved in prostaglandin metabolism (Benson *et al*, 1977; Christ-Hazelhof *et al*, 1976; Chang *et al*, 1987; Meyer and Ketterer, 1987).

The GST's also have a binding activity for a variety of molecules (Litwack *et al,* 1971). A molecule, originally described as ligandin, turned out to be an Alpha class GST (Habig *et al*, 1974). The importance of this ligand binding activity is not known but it may be a reason for the high levels of the enzyme found in liver cytosol since the enzyme can only bind at a stoichiometrical concentration. Its other catalytic functions are inactivated by this binding. The ligand binding can be either covalent or non covalent to a variety of compounds (Litwack *et al*, 1971). Several carcinogens, including 4-aminobenzene and 3-methylcholanthrene, are found to bind covalently to GST. Non covalent ligands include both endogenous and exogenous compounds such as bilirubin, bile acids and penicillin.

The functions of the individual subunits have not been clearly defined *in vitro*. The regulation of the different subunits is complex with different subunits being found in different tissues and at varying levels. GST subunits have been found to be induced by many compounds including

various toxins, substrates and antioxidants (Pearson *et al*, 1983; Talalay *et al*, 1988; Igarashi *et al*, 1987; Listowsky *et al*, 1988). These variations in expression of a large number of different subunits and the lack of knowledge of the *in vivo* substrates make ascertaining the functional role of the GSTs, complicated. The role of GST in the cell is certainly quite complex. Although many of the subunits appear to be capable of having a role in drug detoxification of both the reactive metabolites of xenobiotics and the products of DNA and lipid peroxidation (Chasseaud, 1979; Tan *et al*, 1988; Jensson *et al*, 1986), many also appear to have a role in the normal physiological metabolism of the cell (Benson *et al*, 1977; Christ-Hazelhof *et al*, 1976).

Although in general, the glutathione S-transferase reactions involve detoxification; there are a few instances where compounds become active through the action of GST (Arrick and Nathan, 1984).

I.3. Stress in the environment

Once aerobic respiration had evolved, cells although benefiting from using oxygen to obtain energy, had to develop ways of protecting themselves against the highly reactive by-products. Cells also have the means of surviving various types of radiation, ultraviolet, X-rays and ionising radiation, as well as coping with the huge variety of chemical toxins present in the environment. Some chemical and physical stresses have common mechanisms of damage; for example, heat shock, radiation and some chemicals and are thought to act at least partially through oxidative stress.

The actual mechanisms by which cells are damaged by oxidative stress, heat, radiation and chemical toxins are varied. Some chemicals act

very specifically on a particular enzyme in a pathway, for example cyanide acts in respiration to inhibit oxidative phosphorylation. Other chemicals show more general toxicity perhaps causing more wide spread damage through for example formation of free radicals.

The physical stresses too can cause damage to varying extents. Exposure to UV causes very specific dimerisation of thymidine in DNA. Exposure to heat however causes much more widespread damage throughout the cell. During heat stress there is an increase in activated oxygen species (Lengfelder and Fink, 1989). This suggests that free radicals may be involved at least partially in heat damage.

The mechanisms by which a cell can protect itself are also very varied. The protection mechanism must also be able to cope with a great variety of different toxins and stresses. Also, constitutively synthesising such a variety of proteins that are only needed in the presence of a toxin or stress would be a significant metabolic handicap. Therefore the protection mechanisms are usually only induced in the presence of the toxin or stress. Bacterial cells have evolved a series of stress responses that can be induced by exposure to the stress.

I.3.1. The Stress Response

Cells have developed a variety of mechanisms to survive sub-optimal environmental conditions. Only the simplest mechanisms have been well studied and are fairly well understood; however more mechanisms may exist that are yet not known. The most studied system is the prokaryotic heat shock response. Cells subjected to a non lethal heat shock develop a tolerance to a subsequent normally lethal dose of heat (Gerner and Scheider,

1975; Henle and Leeper, 1976). This response is also induced by other stresses and may be more accurately described as the stress response or adaptive response. All organisms from bacteria to the higher eukaryotes show some form of this adaptive response (for reviews see Nover, 1984; Atkinson and Walden, 1985; Burdon, 1986; Lindquist, 1986; Welch, 1992).

I.3.2. Stress Proteins

When a cell is heat shocked, depending on the severity of the heat shock, there is a decrease in general protein synthesis except for a group of proteins termed the heat shock proteins. These proteins are required for the development of thermotolerance (Henle and Leeper, 1982; Li and Werb, 1982; Landry, 1982; Subjeck, 1982). Deletion of the genes encoding these proteins leads to loss of thermotolerance (Hakawa and Ryu, 1979; Craig and Jacobson, 1984). Mammalian cells that have been injected with antibodies against heat shock proteins lose thermotolerance (Riabowol, 1988).

The stress proteins seem to give the cell protection during both the stress and the recovery period. The stress proteins are homologous from bacteria to mammals. (For reviews of stress proteins see Carper *et al*, 1987, Welch *et al*, 1991; Weber *et al*, 1992.) Heat shock proteins are divided broadly into two classes. The first is the classical heat shock proteins that are expressed at barely detectable or nil levels. Following exposure to heat, expression of these proteins is induced many fold (hsp). The second class of heat shock proteins are constitutively expressed in the normal cell but following heat shock the levels are induced (hsc). This second class includes proteins that are induced by heat but are also regulated by various parameters that affect the cell growth, for example the glucose regulated

proteins (grp). A number of different conditions are known to induce stress proteins including heat, heavy metals, amino acid analogues as well other more physiological situations such as differentiation, entrance to the cell cycle, growth factors, inflammation, oxidant injury and ischaemia (Li and Werb, 1982; Hahn *et al*, 1985; reviewed by Morimoto *et al*, 1992).

Most of the induced proteins, especially the proteins in the latter (hsc) class have not been identified. The functions of some of the heat shock proteins are beginning to be discovered (reviewed by Ang *et al*, 1991). Some appear to be involved in preventing heat denaturation of proteins or in protein folding following denaturation by a stress. These proteins or closely related ones are also involved in protein folding following de novo synthesis, hence the constitutive requirement for hsp's. Another identified function is that of aiding proteolysis. Once proteins become irreversibly damaged, ubiquitin is found to bind to damaged proteins and targets them for protein degradation.

I.3.3. Regulation of the response

The regulation of the adaptive response in prokaryotes is complex. Some genes may be induced by more than one stress. It is also possible that cross resistance may develop as has been shown for cells exposed to hydrogen peroxide, glucose or nitrogen starvation and heat (Christman *et al*, 1985; Storz, 1991). The response to heat and oxidative stress involves some genes that respond specifically to one stress and some genes that respond to both (Morgan *et al*, 1986). These subsets of genes may be induced co-ordinately; for example in *Salmonella typhimurium,* treatment with H_2O_2 leads to induction of about 30 proteins. A single gene controls the

production of a subset of these proteins, some but not all of which, are also inducible by heat shock (Christman *et al*, 1985; Morgan *et al*, 1986; Christman, 1989).

The initiation of the inductive response to stress is not well elucidated. Protein degradation or protein aggregation can trigger the response (Anathan, 1986; Edington *et al*, 1989) but these are not the sole mechanism as low level stress and certain growth conditions also induce heat shock proteins and thermotolerance without significant levels of protein degradation. Damage to other non protein cellular components such as DNA is also known to cause induction of heat shock proteins (McClanahan and McEntee, 1986; reviewed Holbrook and Fornace, 1991).

I.4. The role of GSH in stress

In higher eukaryotes little is known about mechanism and response to oxidative stress. In *S. typhimurium* however there are a variety of proteins induced by oxidative stress. Several are DNA repair enzymes (exonucleases and polymerase I) and some are involved in direct detoxification of radicals (superoxide dismutase, catalase and glutathione peroxidase). Kimball *et al* (1976) demonstrated a lung antioxidant response when rat lung tissues were exposed to hyperoxic conditions. They measured the activities of a variety of enzymes and showed superoxide dismutase, glucose-6-phosphate dehydrogenase, glutathione reductase and glutathione peroxidase activities, as well as levels of reduced glutathione, were all increased. The Alpha class GSTs in the lung have been shown to have peroxidase activity towards lipid hydroperoxides and may therefore have an important role in prevention of lipid peroxidation (Singhal *et al*, 1992).

Of particular interest to our laboratory are the changes noted in the glutathione related enzymes. GSH appears to be involved in protecting cells against stress including heat, oxidative and various chemical stresses. Exposure to heat, ethanol, H_2O_2, radiation or a variety of chemicals results in an increase in GSH levels (see Lee *et al*, 1983; Mitchell *et al*, 1983; Kimbal *et al,* 1985). Exposure of the cells to glutathione depleting agents will reverse this resistance (Dethiners and Meister, 1981). It would seem lower levels of GSH can sensitise cells to stress. The administration of glutathione depleting agents in conjunction with chemotherapy or radiotherapy in the treatment of tumours is currently under investigation (reviewed by van Bladeren and van Ommen, 1991). There is however conflicting data on the involvement of GSH in thermotolerance. In some cases, depletion of GSH will inhibit development of thermotolerance (Mitchell *et al*, 1983; Mitchell and Russo, 1983; Russo *et al*, 1984; Shrieve *et al*, 1986; Shrieve *et al*, 1989). However, depletion of GSH does not always inhibit development of thermotolerance (Konings *et al*, 1985). Freeman *et al* (1988) showed that the effect on development of thermotolerance was dependent on the severity of the stress used to trigger the thermotolerance. The presence of GSH depleting agents inhibited development of thermotolerance when the initial heat shock was at 45°C. With a relatively mild heat shock of 43°C, GSH depletion did not inhibit thermotolerance development. The 45°C heat shock is found to inhibit protein synthesis whereas a 43°C heat shock does not. The depletion of glutathione was found to slow the recovery of protein synthesis and hence indirectly the development of thermotolerance.

Proteins with active SH groups depend on GSH for the maintenance of their reduced form; they may be common targets for both heat and oxidative stress (Lengfelder and Fink, 1989).

Oxidative stress causes a variety of cytotoxic effects (reviewed by Storz, 1992). At low doses, hydrogen peroxide causes DNA strand breaks caused by radical generation in the presence of metals. $O_2^{\cdot -}$ among other things affects amino acid synthesis. The hydroxyl radical ($HO^{\cdot}$) is thought to be the major mediator of cytotoxicity. This radical is a short lived, highly reactive molecule, therefore the location of its toxicity is very dependent on the site of formation. The actual mechanism of cell toxicity, as for most chemical and physical stresses, is not really known. It does cause DNA mutations; lipid peroxidation and membrane damage; and protein damage, making proteins sensitive to degradation. Due to the capabilities of the GSTs for detoxifying some of the products of oxidative stress, and the importance of reduced glutathione in the maintenance of the redox balance of the cell; oxidative stress has been proposed as the major evolutionary driving force behind the emergence of the use of glutathione and it's related enzymes (Mannervik, 1986).

I.5. Agents used in chemotherapy

Many drugs, with a variety of different mechanisms of action, are used in tumour chemotherapy (reviewed by Farmer *et al*, 1985). Some act directly and others require activation to a more reactive metabolite. Often neither the metabolic fate of the drugs used, nor the mechanisms of action are known. Sometimes more than one mechanism of action is attributed to a drug but which of these is relevant *in vivo* is not known.

The wide varieties of drugs have been divided into groups based broadly on structure and functional groups. The alkylating agents include the classical alkylating agents that have a functional alkyl group capable of binding covalently to macromolecules such as DNA and protein. These include the nitrogen mustards, melphalan, chlorambucil and cyclophosphamide. Other drugs that act to alkylate include cisplatin and hydroxyurea. The major cause of death with these compounds is thought to involve the formation of DNA cross links; although inactivation of metabolic enzymes and widespread membrane damage may participate. Nitrosoureas, such as BCNU, are also alkylating agents but also act to inhibit DNA polymerase.

The anthracyclines, adriamycin and daunorubicin, bind to DNA and membranes and generate free radicals as well as acting as alkylating agents. It is not clear which of these mechanisms is important in cytotoxicity. There are therefore several possible drug resistance mechanisms.

The plant alkaloids, vinblastine and vincristine, act through binding to tubulin, causing depolymerisation of the cell's microtubules. They are also Topoisomerase II inhibitors. As they are transported by P-glycoprotein (P-gp), this is a major cause of resistance to these drugs.

The anti-metabolites inhibit specific enzymes. Methotrexate and 5-fluorouracil act to inhibit nucleotide and nucleic acid synthesis. As their mechanisms of action are so specific, resistance can be obtain by altering a single enzyme as in dihydrofolate reductase amplification in methotrexate resistance (Alt *et al*, 1978).

There are a variety of other miscellaneous drugs used commonly in chemotherapy. Cisplatin causes DNA cross linking. Mytomycin acts as an alkylating agent, a DNA cross linking agent and can generate free radicals.

Due to the many different agents, both in terms of their structure and their mechanism of action, there are a variety of ways in which resistance to these drugs can develop (see p2) (reviews Hayes and Wolf, 1990; Borst, 1991).

I.6. Cross resistance to several drugs

Often when tumour cells become resistant to a drug they demonstrate resistance not only to the drug with which they were treated with but also to a variety of other drugs. This is also true of intrinsic drug resistance. One explanation for this multidrug resistance is the overexpression of P-glycoprotein (Riordan *et al*, 1985; Van der Bliek *et al*, 1986; reviewed by Hochhauser and Harris, 1991). This is a plasma membrane protein of M_r 140-180 000. An increased expression of P-gp has been identified in a number of drug resistant cell lines that display cross resistance (Juliano and Ling, 1976; Bech-Hanson *et al*, 1976). High levels of P-gp expression have been found *in vivo* in some drug resistant tumours showing intrinsic or acquired drug resistance (Fojo *et al*, 1987). There are however other examples of cross resistance that do not appear to involve P-glycoprotein (Danks *et al*, 1987; McGrath and Centre, 1987; Moscow and Cowan, 1988; Lai *et al*, 1989). P-glycoprotein is a transport protein which when overexpressed leads to increased efflux of a variety of drugs hence reducing the internal cell concentration of the drug. There are many anticancer drugs such as the alkylating agents that are not transported by this mechanism.

Resistance to these drugs must involve other mechanisms. Also in many multidrug resistant cell lines, which do show elevated P-glycoprotein, the cross resistance patterns vary quite substantially (Mukhopadhyay and Kuo, 1989). There appears to be at least two genes in the family, but it is difficult to account for this variation in cross resistance. It seems likely that other mechanisms are involved. One possible mechanism is a type of adaptive response.

I.7. Adaptive response in preneoplasia

A variety of changes in drug resistance have been notice in preneoplasia models. Carr and Laishes (1981) showed primary rat hepatocyte cultures from rats treated with acetylaminofluorine (AAF) showed resistance to adriamycin. In the transient hyperplastic foci seen in the liver following exposure to carcinogens, a pattern of biochemical changes has emerged (Astrom *et al*, 1983; Kitahara *et al*, 1983; Buchmann *et al*, 1985; Roomi *et al*, 1985). These preneoplastic foci or nodules are formed in response to a variety of different carcinogens following a variety of different exposure regimes. Although the timing and frequencies of appearance of foci vary, the overall nature of the response is surprisingly similar. In the resistant hepatocyte model for example a fairly large number of drug resistant nodules appear within the liver (Farber *et al*, 1979; Farber, 1984). These nodules also have a variety of biochemical changes associated with them; decreased levels of phase I drug metabolising enzymes: increased glutathione levels; increased levels of phase II drug metabolising enzymes including glutathione S-transferase, DT-diaphorase, epoxide hydrolase, γ-glutamyl transpeptidase and UDP-glucuronyl transferase (Roomi *et al*, 1985). After several weeks the majority of nodules lose these biochemical changes

and become indistinguishable from the surrounding liver tissue. An occasional nodule develops into a tumour, but the relationship between the preneoplastic nodules and the neoplastic tumours is not clear. Several different GST subunits as well as γGT have been shown to be induced in preneoplasia liver and lung models (Astrom *et al*, 1983; Kitahara *et al*, 1983; Buchmann *et al*, 1985; Hanigan and Pitot, 1985; Daly *et al*, 1991).

Similar changes have been noticed in an adriamycin resistant tumour cell line established by Batist *et al* (1986), which showed resistance to a variety of other drugs. A number of biochemical changes were observed in this cell line including alterations in drug accumulation as well as changes in the activities of both phase I and phase II drug metabolising enzymes (Cowan *et al*, 1986).

The responses of cells to cytotoxic drugs also show similar changes. The livers of mice primed with a low dose of cyclophosphamide become resistant to further treatment with cyclophosphamide and show increases in GST and γGT activities (Adams *et al*, 1985; Carmichael *et al*, 1986). In some cases, exposure to one form of stress will induce resistance to other forms of stress. Chinese hamster cells, for example, when exposed to either heat or ethanol become tolerant to both these agents as well as to certain cytotoxic drugs such as adriamycin (Li and Hahn, 1978).

The similarities in the above models have prompted the proposal that the changes involving the drug resistance phenotype of cells are caused by an adaptive response similar to that seen in heat shock (Hayes and Wolf, 1988; Hayes and Wolf, 1990). It is important to establish the extent and nature of these similarities and whether they are regulated co-ordinately as part of a general stress response. It may be that in tumour cells the response gets

switched on either in whole or in part perhaps by a mutation or an amplification of one or more genes that can regulate the response, leading to the outgrowth of a drug resistant population of cells.

I.8. Aims

The glutathione S-transferases and γ-glutamyl transpeptidase are induced in a variety of drug resistance models including preneoplasia, drug priming and several drug resistant cell lines. It has been hypothesised that these changes may be part of a general stress response similar to that seen with the prokaryotic adaptive response (Hayes and Wolf, 1988; Lewis *et al*, 1988; Hayes and Wolf, 1990). The aims of the work described in this thesis were:

1) To establish if the glutathione S-transferases are induced as part of an adaptive response to stress.

   a) Are the GSTs regulated by stress protein inducers, such as heat shock and oxidative stress?

   b) Do the other biochemical changes seen in preneoplasia, and drug priming occur in these same stress induced cells?

2) To establish a model for investigating the regulation of a co-ordinated response. Specifically to establish the nature of the overexpression of Alpha class GST and γ-glutamyl transpeptidase proteins in a chlorambucil resistant CHO cell line, previously worked on in the laboratory by Alex Lewis.

CHAPTER II

Materials and Methods

II. MATERIALS AND METHODS

II.1. Materials

II.1.1. Cytotoxic drugs

Adriamycin

M.Wt. 543.54; Doxorubicin; Adr.-HCL formula weight 580 (Sigma); LD_{50} 21.1 mg/kg intravenous (i.v.); soluble in water and alcohol; class I carcinogen; 10 mM stock solution in water.

CDNB

1-chloro-2,4-dinitrobenzene; MWt 202.56; LD_{50} 1070 mg/kg orally; insoluble in water and alcohols; stable; suspect carcinogen; 100 mM stock solution in ethanol.

Menadione

MWt 172.17; water insoluble; light sensitive; LD_{50} 500 mg/kg orally; carcinogen; possible teratogen; 20 mM stock solution in ethanol.

Ethacrynic acid

2,3-(dichloro-4-(2 methylene-butyryl)phenoxy acetic acid; MWt 303.15; LD_{50} 170 mg/kg i.v., 627 mg/kg orally; very sparingly soluble in water; 2 mM stock solution in media.

II.1.2. Cell lines

NCI H322 — Obtained from The National Cancer Institute, Bethesda Md, USA. Bronchioalveolar tumour type thought to be derived from lung Clara cells.

CHO-99 — Obtained from Van der Valk *et al* (1985), and was a result of a stepwise selection protocol with growth in 99% as the selective agent.

CHO-Chl^R — Obtained from Alex Lewis, and were developed by Craig Robson by stepwise selection on increasing doses of chlorambucil (Robson *et al* 1986).

II.1.3. Antibodies

The antibodies used to detect the three classes of GSTs are all polyclonal antisera raised against the major human forms from each class, A1, M1a or M1b and P1. These antisera have all been thoroughly used in both our laboratory and in the laboratory in which they were raised (J.D.Hayes). They have proven very successful in cleanly detecting the GST subunits. As they are polyclonal antibodies there can be some background bands which can be a problem especially when very low levels of the protein are being detected. Polyclonal antibodies are however very useful when detecting classes of proteins which are closely related. To avoid confusion the antisera raised against the human GST subunits have been termed by the antigen subunit nomenclature followed by a two letter code for the specific antiserum. The antisera for the rat GST subunits are simply known by their subunit nomenclature. The antisera used and the antigens they were raised

Table 4: The antisera used to detect GST subunits

	human		rat	
	Antigen	Antiserum	Antigen	Antiserum
Alpha	A1	A1-NM		
Mu	M1	M1-JN	Yo	Yo
	M1a	M1-MR	Yn	Yn
		M1-MY		
	M1b	M1-SH		
		M1-SP		
		M1-ST		
		M1-SL		
Pi	P1	P1-λ	Yf	Yf
Theta			GST-E	GST-E

against are shown in table 4. All the antisera were obtained from J.D.Hayes and are rabbit polyclonal antibodies except for GST-E which was raised in sheep and was originally obtained from B. Jakoby.

II.1.4. GST standards for Western analysis

Alpha class GST	- human GST-A1
Mu class GST	- mouse Yb_1
Pi class GST	- mouse Yf

The purified GST isoenzymes were all obtained from John Hayes and the human liver and lung cytosol protein fractions were prepared by Michael Glancy.

II.1.5. DNA probes

γGT (S1); a cDNA clone, corresponding to a γ-glutamyl transpeptidase mRNA, isolated from a rat kidney library was obtained from M. Manson.

Alpha class GST (pMP37); a cDNA clone from a human liver library corresponding to GST-A1 mRNA, isolated by Julie Moss.

Mu class GST (jt14); a 400bp *Eco*R I fragment of a cosmid isolated from a human genomic library obtained from J. Taylor; corresponds to mu2 (Taylor *et al*, 1991).

Pi class GST; a 1.4 kb fragment isolated from a human genomic DNA library, provided by Theodore Bammler.

II.2. Methods

II.2.1. In vitro cell culture techniques

II.2.1a. Culture conditions

NCI H322 cells and sublines were all grown in RPMI 1640 + 25 mM HEPES (GIBCO). The media was supplemented with 10% heat inactivated foetal calf serum (GIBCO) and 2mg/ml L-glutamine. CHO cells were grown in Ham's F10 (GIBCO) supplemented with 5% heat inactivated foetal calf serum (GIBCO) and 2mg/ml L-glutamine. The antibiotics, penicillin and streptomycin (GIBCO) were added to media at 2units/ml during microtitre plate assays. During normal growth conditions, cells were grown in humidified incubators at 37°C, 5% CO_2 and were fed 3 times per week. During experiments the cells were fed every day.

Cells were passaged when confluent every 1-2 weeks. The cells were rinsed three times with phosphate buffered saline (PBS). The cells were then harvested with equal volumes of 0.2% trypsin and Versene (0.2 mM EDTA) solutions. 5-10% (NCI H322) or 1-5% (CHO) cells were then seeded into a fresh flask.

II.2.1b. Cell line storage

Cells were harvested as for passaging then resuspended in 80% newborn calf serum (GIBCO) 20% DMSO and stored overnight at -70°C, before long term storage in liquid nitrogen. Frozen cells were thawed into

media at 37°C and washed 2-3 times in media before seeding in $25cm^2$ flasks. Media was then changed the following day.

II.2.2. Cytotoxicity assays

The cytotoxicity assay used throughout this thesis is based on an assay developed by Mossman (1983) and modified by Carmichael *et al.* (1987a). The basis of the assay is the estimation of the relative number of viable cells. This is done using the tetrazolium salt, MTT. The colourless MTT is metabolised to give a purple coloured precipitate, the absorbance of which can be measured with a spectrophotometer. The amount of precipitate produced is directly proportional to the number of viable cells.

With this and many other cytotoxicity assays, the difference between cell kill and inhibition of cell growth is not detectable. Two drugs one of which killed 50% of the cells and the other inhibited cell division two fold would not be differentiated by this assay. To establish more precisely the mechanism of the reduction in the number of viable cells a method involving a colony count would be more appropriate. For the purposes of this thesis the exact nature of the toxicity is not important, and the rapid simple method used enable greater numbers of cell lines and drugs to be assayed.

II.2.2a. MTT assay

Cells are plated out in Costar 96 well plates in a volume of 0.1-0.2 ml media. Cells do not grow at the same rate in the outside wells. This may be due to an effect on humidity or pH as the concentration of CO_2 is affected by the humidity. To counteract this the outside wells are not used but are instead filled with an equal volume of sterile water. The 96 well

plates are then placed inside a humid box inside a humidified, 37°C, 5% CO_2 incubator.

The cells are then given the prescribed treatment and left to recover until day 4 or 5. MTT is then added to the cells (25µl of a 2 mg/ml solution). The cells are left for 4-6 hours, then the media and non metabolised MTT are carefully aspirated off, leaving the cell monolayer and the MTT crystals. The crystals are then dissolved in 50 µl DMSO and the absorbance read using an ELISA reader at a wavelength of 500λ. The data are then compiled using the Mackintosh MacReader 2.0 program

II.2.2b. Establishment of the number of NCI H322 cells to use

The number of cells used varies according to the cell division time, the growth characteristics of the cell type and the requirements of the individual assay.

A test MTT assay was carried out in order to establish the cell numbers to use in the assay. A set of 2-fold serial dilution of NCI H322 wild type cells starting from $4x10^4$ were plated out on a 96 well plate. Each dilution was plated into 6 wells. The MTT assay was carried out after 5 days. The results showed that for the NCI H322 wild type cell line the initial seeding numbers should be $1\text{-}2x10^4$ for an assay with a 5 day incubation period.

II.2.3. Protein analysis

II.2.3a. Immunohistochemistry

Cells were seeded directly onto slide flasks (Nunc) at a density of 1-2x10^5 cells per 9cm^2 flask. The slides were left to settle at 37°C for at least 24 hr. They were then given treatment as described. The chambers were then separated from the slides and the slides rinsed 3 times in PBS containing no phenol red. The slides were then fixed in 50% methanol, 50% acetone for 5 minutes before being air dried and frozen at -70°C. When the slides were required they were defrosted through several changes of methanol: acetone at increasing temperatures. The critical step being a slow increase in temperature around 0°C. Each slide was then spotted with 2-5µl of each primary antibody diluted 1:100. The slides were then incubated in a moist atmosphere for 4-12 hours, before being rinsed 4 x 5 min in PBS without phenol red. 2-3ml of second antibody diluted 1:100. The secondary Ab used was, depending on the nature of the primary antibody, donkey anti-rabbit, rabbit anti-mouse or rabbit anti-sheep conjugated to horse radish peroxidase. The presence of the antigen could then be visualised by staining in diaminobenzidine (DAB), enhanced by 3µM $NiSO_4$ for 20 minutes (Adams *et al*, 1981).

II.2.3b. Protein preparations

4x10^7 of each cell subline were seeded into 175 cm^2 flasks. After 48 hours the cells were harvested using trypsin. The cells were washed twice in cold PBS then resuspended in 1.5 ml cold buffer H (10mM NaH_2PO_4 pH8; 2mM $MgCl_2$; 2 mM DTT; 1 mM EDTA), left 10-20 minutes on ice, then

homogenised in a glass teflon homogeniser until 90-95% of the nuclei were released. The suspension was then spun twice at 1000g for 5 minutes to separate the nuclei. The supernatant was then processed through 10000g and 100 000g spins to produce an S_{100} fraction. The nuclei were further purified through a ficoll cushion. The crude nuclear fraction was resuspended in 0.5 ml buffer H, and mixed with 1.5 ml buffer H + 20% ficoll. The suspension was then layered on to a 4 ml buffer H + 20% ficoll cushion and spun at 5000g. The spun nuclei were then washed with buffer H and resuspended in 100µl buffer H. The samples were kept on ice at all times wherever possible. The low speed spins were carried out in a microcentrifuge in the cold room; the high speed spins were performed at 2°C.

II.2.3c. Protein Estimation

Protein estimations were made following the method of Lowry *et al.* (1951). A volume of 1 ml alkaline copper solution (O.5ml of 40mM $CuSO_4$, O.5ml of 71mM NaK tartrate, and 49 ml of 70mM $NaCO_3$: $5H_2O$, 40mM NaOH, made fresh) was added to 5-20µl of each protein sample and vortexed. Assays were done in triplicate. After 10min 100µl of stock Folin Ciocalteau reagent diluted 1:1 with distilled water was added. After 30 minutes, absorbencies of the standards (a range of dilutions of BSA from 0-250µg) were measured at 600nM and a standard curve was generated. The absorbencies of the samples were then measured and the protein concentration determined.

When numerous samples were to be measured or the protein sample was low, protein estimations were carried out in 96 well microtitre plates.

Estimations were carried out as above except using $^1/_{10}$ volumes and in quadruplicate. Absorbencies were measured using an ELISA reader and the data processed using the MacReader 2.0 program on an Apple Macintosh computer.

II.2.3d. Western blot analysis

Crude cellular protein extracts were fractionated by electrophoresis using polyacrylamide gels containing the denaturant SDS by the method of Laemmli (1970). A discontinuous gel system was used; the separating gel was composed of the following components in a volume to pour one gel for the Protean II (BioRad) system: 10.7ml 40% Acrylamide; 7.4ml 2% bis-acrylamide; 9.25ml separating buffer (1.5M Tris-HCl, pH8.8, 0.5% SDS), 2ml 1% ammonium persulphate; 20µl TEMED in a total volume of 37ml that gives a final concentration of acrylamide of 12%. The stacking gel was composed of 1.1 ml 40% Acrylamide; 0.7ml 2% bis-acrylamide; 2.5ml stacking buffer (0.5M Tris pH6.8, and 0.5% SDS); 0.3ml 1% ammonium persulphate, and 10µl TEMED in a total volume of 10ml. The running buffer contained at a 10X concentration 126g Tris base, 80g glycine, 20g SDS in a total volume of 2l. The proteins were denatured in 1x boiling mix (10% stacking buffer, 2% SDS 5% β-mercaptoethanol, 10% glycerol and 0.005% bromophenol blue) by placing in boiling water for 5 min. The proteins are run at constant amperage; 40mA/gel. Protein molecular weight markers were the Rainbow markers (molecular weights of 69k, 46k, 30k, 21.5k, 14.3k) [Amersham].

The fractionated proteins were transferred to a nitrocellulose membrane using the method of Towbin *et al* (1979) by electroblotting in

buffer (20mM Na_2HPO_4, 20% methanol) at 250mA overnight. The nitrocellulose is then washed twice in TBST (9g/l NaCl, 6g/l Tris, 0.5ml Tween-20) each for 10min, blocked in TBST + 3% BSA for 1-2hr, washed twice in TBST and the first antibody raised against the protein of interest is added usually at 1:500 dilution. This is incubated at room temperature for 1hr. The membrane is then washed four times for 15min each in TBST. The second antibody (donkey anti rabbit antibody conjugated to horseradish peroxidase [SAPU]) is added in TBST for 1hr at 1:1000 dilution to detect the first antibody complex. This is then washed 4X as before. Peroxidase activity is detected by the addition of substrate (120mg of 4-chloro-1-napthol in 40ml methanol added to 200ml TBS [9g/l NaCl, 6g/l Tris pH7.9] with the final addition of 80µl H_2O_2) and allowed to develop until desired signal is detected. Further radioactive detection of the antibody complex was carried out by incubating the membrane in 50ml TBST containing 50µl Protein A labelled with ^{125}Iodine [Amersham] (Ochs *et al*, 1983). Except where stated Western blot analysis was carried out at least twice.

II.2.4. Nucleic acid analysis

II.2.4a. Plasmid DNA preparations

A bacterial colony harbouring the plasmid of interest was inoculated into 500ml of L-broth plus 100µg/ml ampicillin and grown overnight at 37°C while shaking. The bacteria were spun down at 8000rpm for 10 min and the pellets were resuspended in 5ml cold GTE (50 mM glucose, 50 mM Tris pH7.5, 10 mM EDTA) plus 1mg/ml lysozyme. After 15 min on ice 20ml of alkaline/SDS solution (0.25M NaOH, 1% SDS) was added and allowed to stand for 10 min. Then, 15ml of high salt solution (3M potassium

acetate, 2M glacial acetic acid, pH4.5) was added to precipitate chromosomal DNA. The supernatant was filtered through muslin and precipitated by the addition of 0.6 volume of isopropanol. The DNA (plus contaminants) was pelleted at 3000rpm for 10 min, and the resulting pellet redissolved in 13.4ml of TE (10 mM Tris-HCl pH7.6, 1 mM EDTA). The preparations were then prepared for banding by density gradient centrifugation. After addition of 14.8 grams of CsCl and 800µl ethidium bromide (10mg/ml), the samples were centrifuged in the TV865B rotor at 40K rpm for 20 hr. The supercoiled plasmid band was removed with a syringe and either precipitated twice with ethanol or the ethidium bromide was removed by butanol extraction and the DNA dialysed overnight against a litre of TE.

II.2.4b. Genomic DNA preparations

Genomic DNA was isolated from cell pellets from a single $75cm^2$ flask by lysing cells in 2ml STNE (0.5% SDS, 150mM NaCl, 10mM Tris 10 mM EDTA pH7.5). The lysate was made 100µg/ml RNase A (Sigma) and incubated for 1hr at 37°C. Proteinase K was then added at a concentration of 250µg/ml and incubated for a further 4hr at 37°C. This solution was then extracted in one volume phenol (saturated with TE pH8.0), then in one volume phenol: chloroform: isoamyl alcohol (25:24:1) and finally in one volume chloroform. DNA was precipitated using 0.5 volume 7.5M ammonium acetate and 2 volumes absolute alcohol and the precipitate spooled out with a glass rod. This is then washed in 60% ethanol containing 2.5M ammonium acetate and the DNA dissolved in TE.

II.2.4c. RNA preparations

RNA was isolated from cultured cell by the method of Birnboim (1988). Confluent cultures of cells in 75 cm^2 flasks were harvested into 4 ml cold RES (0.5M LiCl, 1M Urea, 0.25% SDS, 0.02M Sodium Citrate, 2.5mM EDTA, pH6.8). In a universal tube the lysate was sonicated at low power (10-14μ) for 5-10s. The addition of 150μl of proteinase K (1mg/ml) was followed by incubation at 50°C for 30min. RNA was selectively precipitated by the addition of 1/20th volume of 2M sodium acetate pH5.2 and 1.5 volumes of cold absolute ethanol and allowed to stand at -20°C for 20 min. Centrifugation at 10K rpm for 10 min pelleted the RNA. The RNA was furthered purified by dissolving the pellet in 1 ml RES and extracting with 1/10th volume chloroform. The aqueous phase was removed and 1/100th volume 2M acetic acid and 1 volume of LiCl/ethanol (3 volume 5M LiCl: 2 volume ethanol) was added and left to precipitate overnight at 0°C. RNA was pelleted as before and finally dissolved in 200μl CCS (1mM sodium citrate, 1mM CDTA, 0.1% SDS, pH 6.8). The concentration of RNA was determined spectrophotometrically at 260 μm.

II.2.4d. Southern blot analysis

Genomic DNA was stored at -20°C until required. Routinely 5μg of DNA per gel lane was digested with a restriction enzyme carried out according to manufacturers' instructions. Agarose gels were subjected to electrophoresis in 1X TAE buffer (40mM Tris-HCl pH8.2, 20mM Sodium acetate, and 1mM EDTA). Digested DNA was fractionated on 0.8% agarose on 20x24cm gels run at 40V overnight (> 16hr). At the end of the run the DNA was stained with ethidium bromide for 15min, rinsed, photographed,

and left on the UV transilluminator for 2-3 min to partially cleave the DNA for more efficient transfer of high molecular weight DNA. DNA was transferred and immobilised onto Hybond-N by the method of Southern (1975). The DNA was denatured *in situ* by soaking the gel in Denaturing Solution (0.5M NaOH, and 1.5M NaCl) for 45min and then the gel pH neutralised in Neutralising Solution (2M NaCl, 1M Tris-HCl pH 5.5) for 45 min. The DNA was transferred onto Hybond-N membrane by capillary action overnight by drawing 20X SSC (3M NaCl, 0.3M tri-sodium citrate) through the gel with absorbent paper towels. The DNA was permanently fixed onto the membrane using UV from the transilluminator for 2.5-4min exposures and further by baking for 1hr at 80°C.

The genomic DNA fragments of interest were detected by hybridisation to a complementary radiolabelled probe. The membrane was first prehybridised in 5X SSC, 4X Denhardt's Solution (20x solution is 2g Ficoll [Type 400, Pharmacia], 2g polyvinylpyrrolidine, 2g BSA [fraction V; Sigma]), 10% Dextran Sulphate, 0.1% SDS, 0.1% sodium pyrophosphate (NaPPi) at 68°C for at least 1 hr. The denatured radiolabelled probe was then added and allowed to hybridise overnight at the same temperature. The blot was then washed free of unhybridised probe with 3 washes (of 10-20 min) of 2X SSC 0.1% SDS, 0.1% NaPPi. The blot was then exposed to X-ray film (Kodak X-AR) overnight or longer at -70°C.

The blots could be re-used for hybridisation to a different probe by melting off the previous probe in a boiling solution of 0.2% SDS, 0.1% NaPPi for 10 min.

The DNA probes used for detection of complementary sequences with both Northern and Southern blot analyses were radiolabelled by random

priming by the method of Feinberg and Vogelstein (1983). Specific restriction fragments of DNA were isolated from the plasmid vector by electrophoretic fractionation on agarose gels. The specific DNA fragment was identified and that region of the gel was cut out. The DNA was separated from the agarose by Elu-Tips (Schleicher & Schuell) by the manufacturers' instructions. The DNA was then labelled with ^{32}P-CTP (3000Ci/mmole) (Amersham)using the Random Primed DNA Labelling kit (Boehringer Mannheim) following manufacturers' instructions.

II.2.4e. Northern blot analysis

RNA from the cell culture preparations was fractionated by electrophoresis on denaturing 1.5% agarose gels. The RNA was denatured at 50°C for 10 min in 50% formamide, 18% formaldehyde [40% solution], and 1X MOPS Buffer (20 mM MOPS pH7, 5mM Sodium acetate, 5mM EDTA). To enable visualisation of the RNA after electrophoresis, 1µl of 0.1mg/ml ethidium bromide was added to each sample. Relative loading of the lanes was checked by UV illumination. The gel and running buffer contained 1X MOPS buffer. In addition the gel contained 18% formaldehyde [40% solution]. The gel was run overnight at 30V, then soaked in 10X SSPE (20X SSPE is 3.6M NaCl, 0.2M NaH_2PO_4, 0.2M EDTA, pH7.4) for 10 min. The RNA was transferred to Hybond-N by capillary action as for the Southern analysis described above, however 10X SSPE was used as the transfer solution.

Hybridisation and detection of radiolabelled probe were performed as for Southern analysis except that SSPE was used in place of SSC.

II.2.4f. PCR

A specific RNA species was amplified as a DNA fragment by the method of reverse transcriptase polymerase chain reaction (RT-PCR) as described by Kawasaki (1990). RNA amplification is done in two steps; the first is the production of cDNA which is, secondly, amplified by the polymerase chain reaction. One microgram of total RNA was used in each reaction in a volume of 20µl. cDNA was made in 1XPCR Buffer (50mM KCl, 20mM Tris-HCl pH8.4, 2.5mM $MgCl_2$, and 0.1mg/ml bovine serum albumin) containing 1mM each of the dNTPs (100mM stock solutions from Pharmacia diluted in TE), 1 unit/µl RNAsin [Promega] 100pmoles random hexamers (solution in TE)[Pharmacia] and 200 units of MoMLV reverse transcriptase [BRL]. The samples were incubated at 20°C for 10 min and then switched to 43°C for the remaining hour. They were then heated for 5 min at 95°C and quick chilled to denature the RNA-DNA hybrids. For the PCR amplification, 80µl of 1XPCR Buffer was added along with 50 pmoles of two specific oligonucleotide primers. To amplify the Mu class mRNAs specifically, the following oligonucleotides were used TGAC(A/C)GAAGCCAGTGGCTGA and CTTGT(C/T)(C/T)CCTGC(A/G)AACCATGG. One unit of *Taq* polymerase (Promega) was added and the solution was layered with 100mλ of mineral oil. The thermal cycle profile used was 1) denaturing at 95°C for 30s, 2) annealing at 55°C for 30s and 3) incubating for 30s at 42°C and cycled for 35 rounds. The amplified product was analysed by electrophoresis on agarose gels and visualised with ethidium bromide under UV transillumination.

CHAPTER III

Preliminary analysis of GST in various stress models

III. PRELIMINARY ANALYSIS OF GST IN VARIOUS STRESS MODELS

To establish if the GSTs were regulated co-ordinately and to establish which models might be worth investigating further; the influence of stress on the GST levels was assayed using immunohistochemistry as a preliminary screen. This assay has advantages in speed and simplicity and enables a number of antibodies to be used simultaneously. The small numbers of cells needed and the speed of the assay enable many variations in conditions, as well as different exposure and recovery times, to be assayed with a manageable amount of cell culture and preparation. The basic method involves culturing the cells on slide flasks that allow the cells to be fixed directly onto the slides. Each antibody can then be spotted onto the fixed cell monolayer and the interaction detected with a second antibody, linked to horse radish peroxidase in combination with its substrate, diaminobenzidine (DAB). A variety of transient and permanent stress resistant models were fixed onto slides and are described below.

1) A Chinese hamster ovary cell line, from Van der Valk *et al* (1985) which had been selected to be resistant to 99% O_2 (CHO-99) was assayed along with the original wild type cells (CHO-20). These oxygen sensitive cells were also exposed to 98% O_2 for 48 hours before fixation.

2) A chlorambucil resistant Chinese hamster cell line (CHO-Chl^R) was assayed along with its progenitor cells (CHO-K1). This cell line was developed by Robson *et al* (1986) and has been studied in our laboratory.

3) NCI H322 cells were exposed to heat shock by the following method. Upon seeding, the cells were left to recover for 24 hours, they were

then placed at 42°C for 48 hours, after which they were left to recover for 1, 3 or 5 days at 37°C.

All the cells were plated out at 1 x 10^5 cells in 9 cm^2 slide flasks and grown at 37°C, 5% CO_2, 100% humidity unless otherwise stated. The CHO-20 and CHO-99 cell lines were grown in either 20% or 98% O_2, 2% CO_2. The cells were then fixed in 1:1 methanol: acetone and the slides stored at -70°C.

To assess the effects on proteins involved in drug resistance and the proposed co-ordinate stress response; the cells were assayed by immunohistochemistry using antisera raised against glutathione S-transferases, cytochrome P450's, topoisomerase II and a variety of other proteins (see table 5 legend). The levels of protein within the samples were estimated by eye and scored on a basis of - to ++++.

Many of the cell samples showed very little change with most of the assayed antibodies as compared to their controls (table 5). However changes were detected in the chlorambucil resistant cell line (CHO-Chl^R) showing increases in Alpha, Mu and Pi class GSTs. Also of note was the presence of staining with the antibody to the heat shock protein, hsp 70 (antibody N27). This was found in both the chlorambucil resistant (CHO-Chl^R) and sensitive (CHO-K1) cell lines and is not found to differ significantly between the two lines. It was not detected in all CHO cell lines as the CHO-20 cell line was negative for staining with both the hsp antibodies (N27 and CG2). The presence of hsp70 may be inherent in the CHO-K1 cell line and its derivatives, and since it did not change significantly between these lines this was not pursued further.

Table 5: Results of immunohistochemical analysis.

All cells were seeded at $1x10^5$ cells per 9 cm^2 slide flask. NCI H322 cells were exposed to 42°C for 2 days (2d 42°C) and then allowed to recover for 2 hours (+2hr 37°C), 1 day (+1d 37°C), 3 days (+3d 37°C) or 5 days (+5d 37°C). Control NCI H322 cells were grown for 3 days at 37°C (3d 37°C). A Chinese hamster ovary cell line resistant to chlorambucil (CHO-Chl) and its chlorambucil sensitive progenitor cell line (CHO-K1), were grown in the absence of chlorambucil. A Chinese hamster ovary cell line resistant to 99% oxygen (CHO-99) and its oxygen sensitive progenitor (CHO-20) were grown in an atmosphere of 20% O_2, 2% CO_2. The oxygen sensitive cells were also exposed to 98% O_2, 2% CO_2 for 48 hours (CHO-20 [98]). Cells were fixed in 50% methanol, 50% acetone. Immunohistochemical analysis was carried out using antibodies to cytochrome P450's (CYP2C6, CYP3A1, CYP2B1, CYP1A2, CYP1A1), the three major human glutathione S-transferase isoenzymes (GST-A1, GST-M1, GST-P1), γ-glutamyl transpeptidase (γGT), P-glycoprotein (P-gp), heat shock protein 70 family (N27 and CG2), oncogenes (c-Ha-ras, c-myc and p68) nuclear laminin (LN43) and topoisomerase II (A17). Immunohistochemical analysis was carried out as described in chapter II (p40). Staining was visualised using a second antibody linked to horse radish peroxidase and its substrate diaminobenzidine). The nuclear laminin antibody (LN43) was linked to a fluorescent dye and was visualised under fluorescent microscopy. The location of the staining was either cytoplasmic (cyt) or nuclear (nuc). Those antibodies expected to stain for membrane proteins are marked as such. Results are scored on a scale of: - to +++++; No visible staining (-); staining barely visible (+/-); intense staining (+++++).

			3dy 37C	2dy 42C + 2hrs 37C	2dy 42C + 1dy 37C	2dy 42C +3 dy 37C	2dy 42C + 5dy 37C	CHO-K1	CHO-Chl	CHO-20	CHO-20 (98)	CHO-99	
PIS			+/-	+/-	+/-	+/-	+/-	-	-	+/-	+/-	-	PIS
P-450	CYP2C6	cyt	+/-	+/-	+/-	+/-	+/-	-	-	+/-	+/-	+/-	PB1a
	CYP3A1	cyt	++	++	++	++	++	++	++	++	++	++	PB2a
	CYP2B1	cyt	+/-	+/-	+/-	+/-	+/-	+/-	+/-	+/-	+/-	+/-	PB3a
	CYP1A2	cyt	+	+	+	+	+	++	++	+	++	++	MC1a
	CYP1A1	cyt	+/-	+/-	+/-	+/-	+/-	+	+	+	++	+	UT1
GST	Alpha	cyt	+ to ++	+ to +++	+ to ++	+ to ++	+ to +++	+	++	++	++	+	Alpha
	Mu	cyt	+	+	++	+	+++	++	+++	+++	+++	+	Mu (cytosol)
	Mu	nuclear	++	+++	++++	++	+++++				++		Mu (nuclear)
	Pi	cyt	+ to ++	+ to +++	+ to +++	+ to ++	+ to ++++	-	+	+	+	+	Pi
GGT	GGT	membrane	+/-	+/-	+/-	+/-	+/-	+/-	-	+/-	+/-	-	GGT
P-gp	Ling	membrane	+/-	+/-	+/-	+/-	+/-	-	-	+/-	-	-	Ling
	MRK16	membrane	+/-	+/-	+/-	+/-	+/-	+/-	-	+/-	-	+/-	MRK16
hsp70	N27	cyt	+	++	+++	++	+	+	+	+/-	-	+/-	N27
	CG2	cyt	+	+++	++++	++	++	-	+/-	+/-	-	-	CG2
ras			+/-	+/-	+/-	+/-	+/-	+	+/-	+/-	+	+	ras
myc			+/-	+/-	+/-	+/-	+/-	+/-	+/-	+/-	+	+	myc
p68	204	nuclear	++	++	+	++	++	+	+	+	+	+	204
laminin	LN43	nuclear	+++	++++	+++	+++	+++	++	++	++	++	++	LN43
Topo II	A17	nuclear	+ to ++++	+ to ++	+	+ to +++	+ to +++++	++	++	++	+	++	A17

The oxygen resistant cells (CHO-99) showed few changes in the protein levels assayed, with the exception of decreases in the levels of Alpha and Mu class GST relative to the oxygen sensitive CHO-20 cells. In contrast, when the oxygen sensitive cells were exposed a to high percentage of oxygen (CHO-20 [98]), the levels of Mu class GST increased. This was nuclear whereas other Mu class staining in these cells, including that in the oxygen resistant cells, was cytoplasmic. The nuclear staining might be due to break down of the nuclear envelope and leakage of protein into the nucleus, but as the other cytoplasmic proteins assayed were not found in the nucleus and there was no corresponding loss of nuclear proteins (LN43) in these cells, this seemed unlikely. When the oxygen sensitive cells were exposed to high levels of oxygen, the levels of topoisomerase II decreased. The levels of topoisomerase II within a cell are regulated by the cell cycle (Heck *et al*, 1988) and so are found at high levels in only a proportion of cells, within a population. The proportion of highly expressing cells varies depending on the numbers of cells undergoing division within the population; a rapidly dividing population will have a higher proportion of cells with high levels of topoisomerase II. The CHO-20 cells when exposed to 98% O_2 would be expected to stop dividing hence the expected low level of topoisomerase II found in the population. The wild type cells survive but did not appear to divide for at least 2 days. After about 3-4 days, the cells started to lift off and die.

III.1. Changes in protein expression following NCI H322 heat shock.

The greatest changes in protein levels occurred in the GSTs after heat shock of the NCI H322 cells (table 5). Light micrographs of the immunohistochemical staining for topoisomerase II and Alpha, Mu and Pi

class GSTs are shown in figure 1. The immunohistochemical staining of untreated wild type NCI H322 cells with Alpha and Pi class GSTs showed staining within the cytoplasm as expected. The immunohistochemistry with the Mu class GST showed staining within the nucleus of the cell. As the samples are whole cells rather than sections it can be difficult to establish the exact location of the staining within the cell using normal bright field light microscopy. For this reason the slides were also studied using a confocal microscope. The confocal microscope uses a laser light of a specific wavelength that results in a very narrow depth of focus, enabling a narrow planar section to be easily visualised. The confocal micrographs shown in figure 2 confirmed that the Mu class GST staining was located solely in the nucleus. The levels of this nuclear Mu class GST staining are increased in the cells kept for 2 days at 42°C. The levels then decrease in the period immediately following the return to 37°C before a substantial increase is seen on day 5 recovery.

Staining of the Alpha class isoenzymes was present in the cytoplasm of untreated NCI H322 cells. Alpha class GST levels changed little with the 42°C heat shock. The staining was however, variable between individual cells within a population. The levels were generally low and more homogeneous between cells in the control samples. The levels did appear to increase slightly on day 1 and day 5 during the recovery period at 37°C.

Pi class GST was present at easily detectable levels in the cytoplasm of the wild type cells (figure 2). After heat shock the levels appear to drop slightly, before recovering and increasing to well above the control cell levels (figure 1). The temporal pattern was similar to that seen with the nuclear Mu class GST staining.

Figure 1: Light micrographs of heat shocked NCI H322 cells stained for Alpha, Mu and Pi class glutathione S-transferases and topoisomerase II described in Table 5.

NCI H322 cells were seeded at 10^5 cells per 9 cm^2 slide flask. Cells were then exposed to 42°C for 2 days (2d 42°C) and then allowed to recover for 2 hours (+2hr 37°C), 1 days (+1d 37°C), 3 days (+3d 37°C) or 5 days (+5d 37°C). Control cells were grown for 3 days at 37°C (3d 37°C). Cells were fixed and stained for Alpha, Mu and Pi class GSTs and topoisomerase II as described in table 5. Samples were photographed under normal bright field light microscopy (x100).

Immunohistochemistry of heat shocked NCI H322 cells.

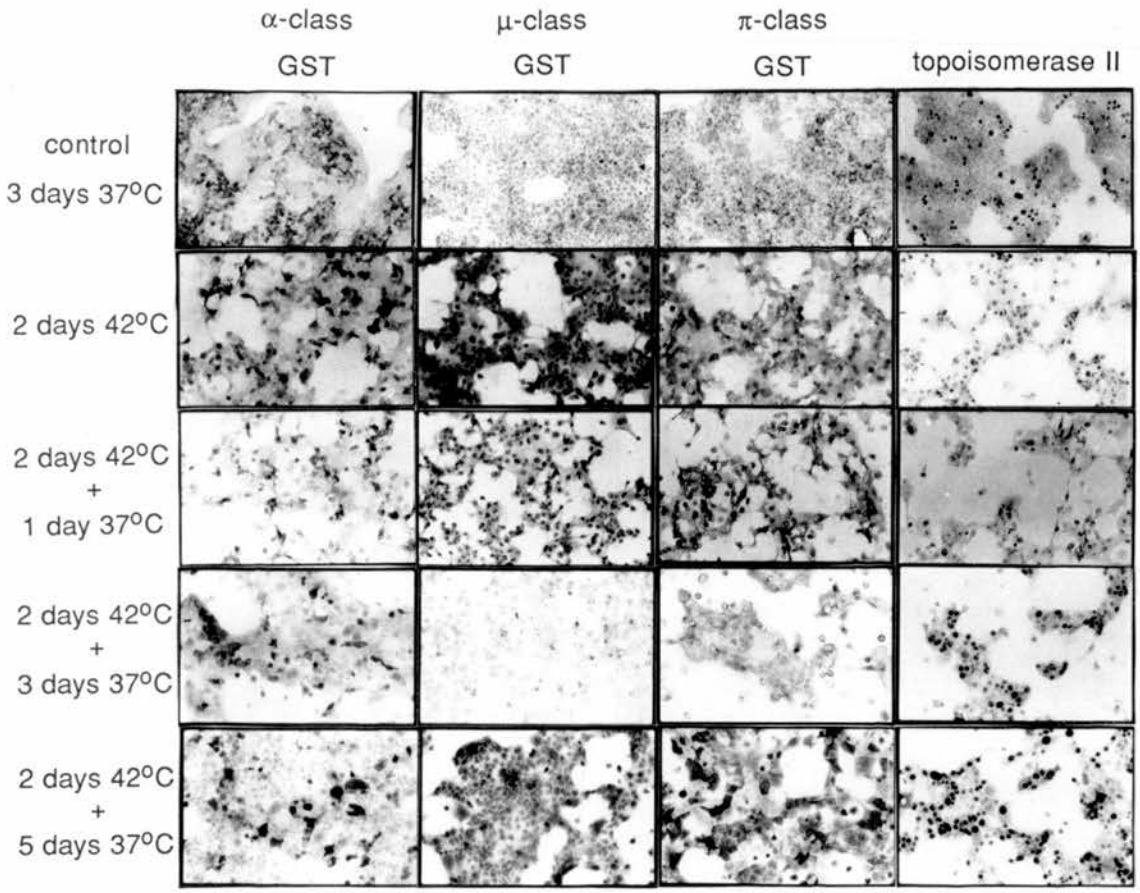
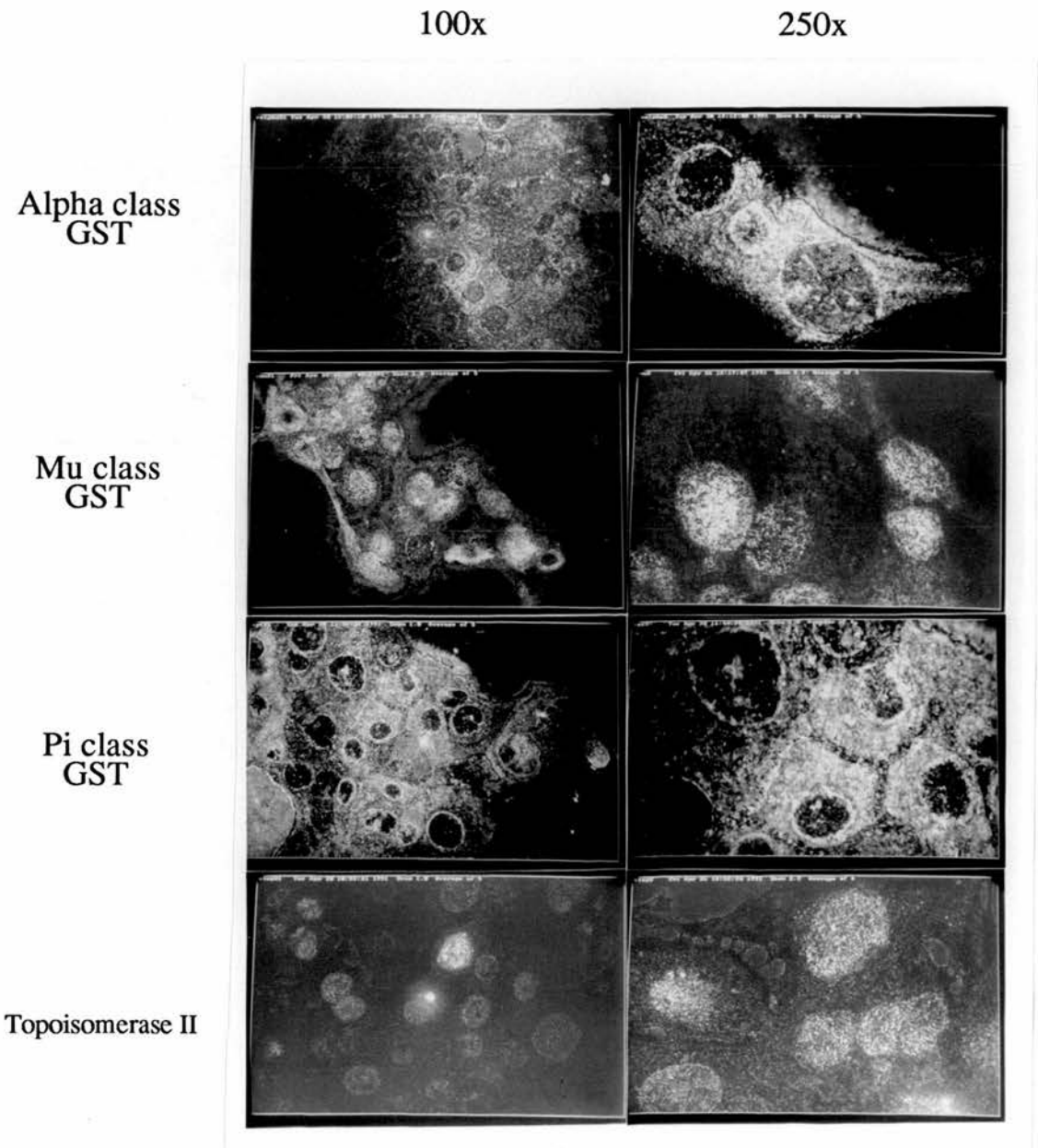


Figure 2: Confocal microscopy of Alpha, Mu and Pi class GST and topoisomerase II immunohistochemistry staining in heat shocked NCI H322 cells.

NCI H322 cells were seeded at 10^5 cells per $9cm^2$ slide flask. Cells were exposed to 42°C for 2 days and then allowed to recover for 5 days. Cells were fixed in 50% methanol, 50% acetone. Immunohistochemical analysis was carried out using antibodies to the three major human glutathione S-transferase isoenzymes (GST-A1, GST-M1, GST-P1) and topoisomerase II (A17). Immunohistochemical analysis was carried out as described in chapter II (p40). Staining was visualised using a second antibody linked to horse radish peroxidase and its substrate diaminobenzidine). Samples were photographed under confocal microscopy at 100x and 250x. Cellular location of staining in these samples is representative of other periods during heat shock and recovery as well as in the control cells grown at 37°C.


100x
250x
Alpha class GST
Mu class GST
Pi class GST
Topoisomerase II

Of the cytochrome P-450 antibodies, only CYP3A1 showed any staining at all and this was fairly uniform throughout both the heat shock and the recovery periods. Neither of the antibodies raised against P-glycoprotein or the gamma-glutamyl transpeptidase antibody showed any change in staining. The level of staining was very low.

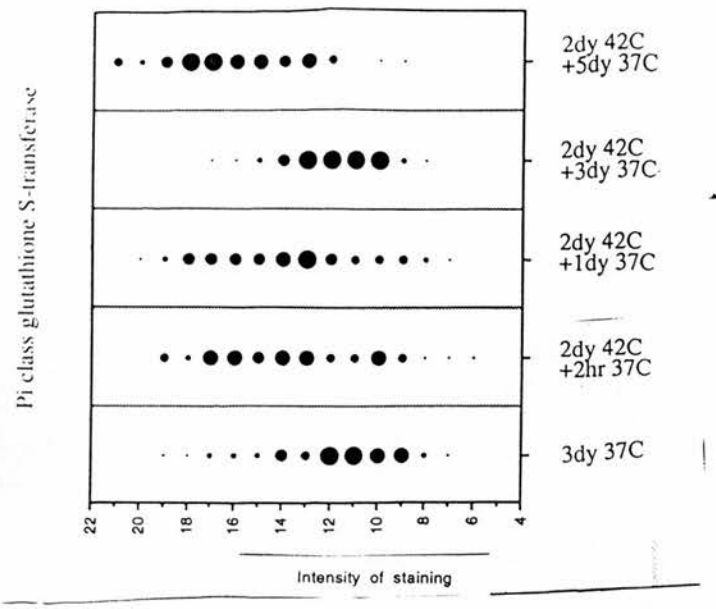
Topoisomerase II showed a decrease in staining immediately after heat shock followed by increases to much higher than the control levels. The variation between individual cells was great with some cells expressing very low levels and some very high.

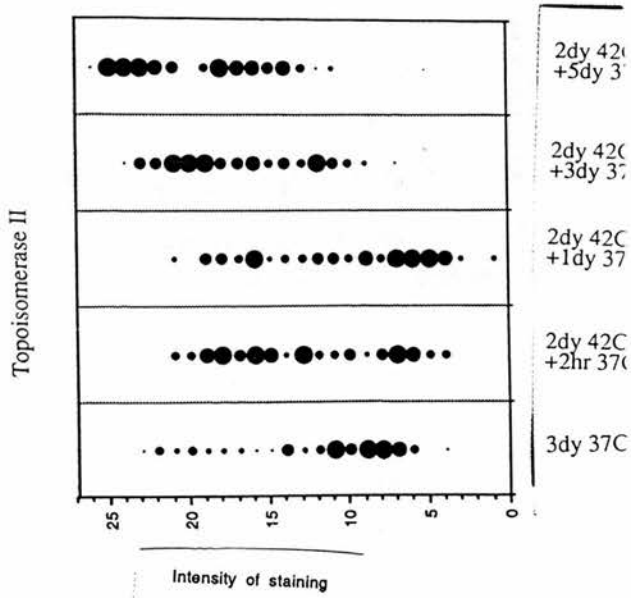
The heat shock samples were analysed using an image analyser to establish the various proportions of cells with a particular density of staining within the population. It was also thought this would give a more accurate portrayal of the results than the estimate by eye. The results are shown in figure 3 and confirm the results scored by eye in table 5. The cytochrome P-450, CYP3A1 showed no overall change in the intensity of staining within the population. Alpha-class GST showed little change in the average intensity of staining, although it appeared that the staining within the cell population was more heterogeneous during the recovery period. Some cells showed more intense staining and some less intense staining within the population. The Mu-class GST showed more uniform staining throughout the population that increased after 2 days heat shock and 1 and 5 days' recovery (as shown in table 5). Pi-class GST showed a similar temporal pattern to the nuclear Mu class staining, although the staining pattern in the cytoplasm also became more heterogeneous, with not all cells within the population showing increased Pi-class GST staining.

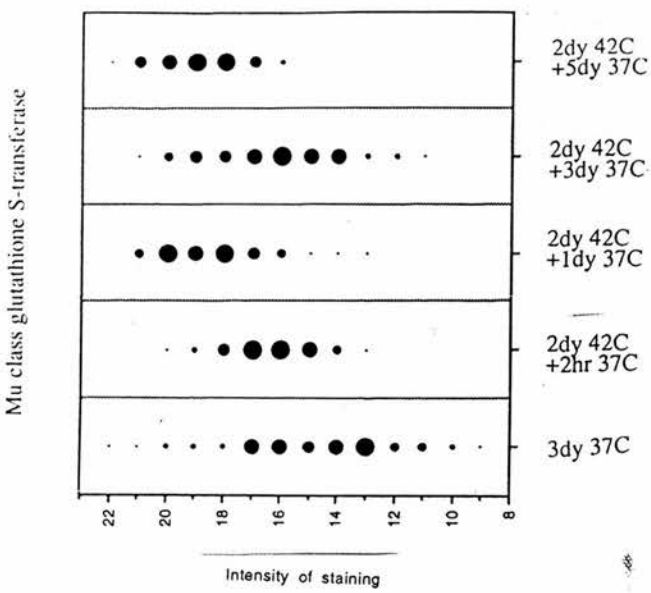
Figure 3: Densitometry of immunohistochemistry results for heat shocked NCI H322 cells.

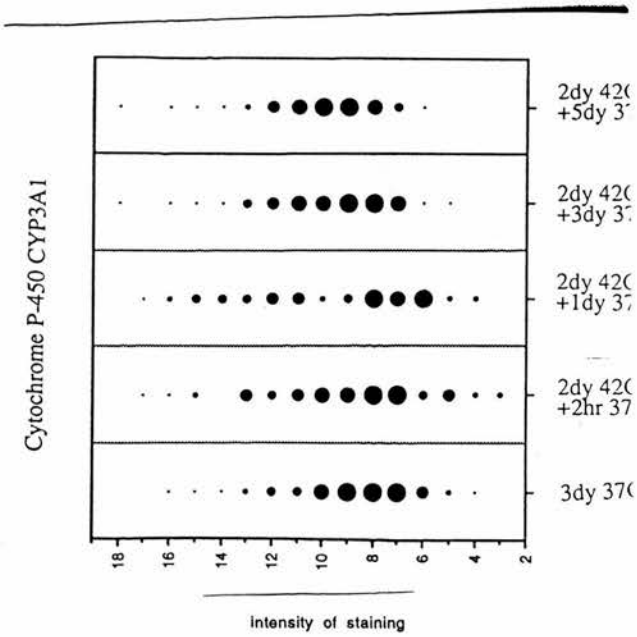
The heat shocked cells stained for topoisomerase II, cytochrome P-450 CYP3A1, and Alpha, Mu and Pi class glutathione S-transferases as described in table 5 were subjected to image analysis to determine the intensity of staining for individual cells within the whole population.

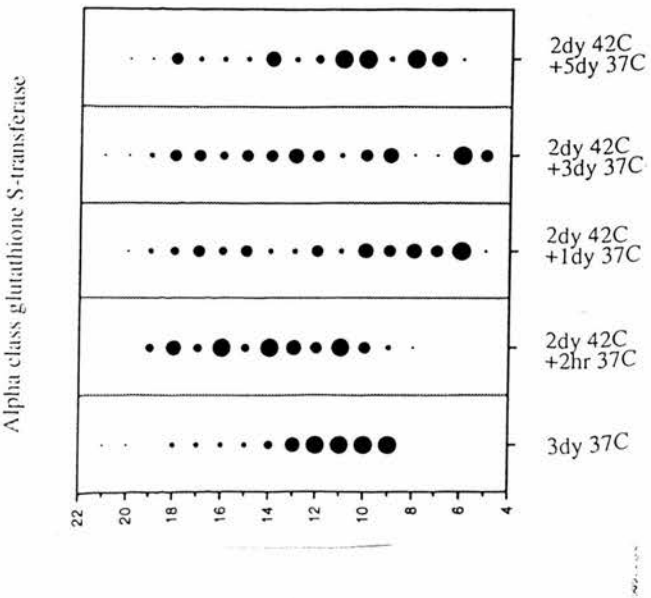
NCI H322 cells were seeded at 10^5 cells per 9 cm^2 slide flask. Cells were exposed to 42°C for 2 days (2d 42°C) and then allowed to recover for 2 hours (+2hr 37°C), 1 days (+1d 37°C), 3 days (+3d 37°C) or 5 days (+5d 37°C). Control cells were grown for 3 days at 37°C (3d 37°C). Cells were fixed and stained for Alpha, Mu and Pi class GSTs and topoisomerase II as described in table 5. From each sample, 200-300 individual cells were analysed. For each sample the numbers of cells with each intensity of staining are shown by the size of the dot.


Pi class glutathione S-transferase
2dy 42C +5dy 37C
2dy 42C +3dy 37C
2dy 42C +1dy 37C
2dy 42C +2hr 37C
3dy 37C
22 20 18 16 14 12 10 8 6 4
Intensity of staining


Topoisomerase II
2dy 42C +5dy 3
2dy 42C +3dy 3
2dy 42C +1dy 37
2dy 42C +2hr 37C
3dy 37C
25 20 15 10 5 0
Intensity of staining


Mu class glutathione S-transferase
2dy 42C +5dy 37C
2dy 42C +3dy 37C
2dy 42C +1dy 37C
2dy 42C +2hr 37C
3dy 37C
22 20 18 16 14 12 10 8
Intensity of staining


Cytochrome P-450 CYP3A1
2dy 42C +5dy 3
2dy 42C +3dy 3
2dy 42C +1dy 37
2dy 42C +2hr 37
3dy 37C
18 16 14 12 10 8 6 4 2
Intensity of staining


Alpha class glutathione S-transferase
2dy 42C +5dy 37C
2dy 42C +3dy 37C
2dy 42C +1dy 37C
2dy 42C +2hr 37C
3dy 37C
22 20 18 16 14 12 10 8 6 4
Intensity of staining

The intensity of staining for topoisomerase II in the control cells is probably indicative of the proportion of cells undergoing cell division at any one time as suggested above (Heck *et al*, 1988). In the control cells the majority had low levels. The few cells that had very high levels probably represented those in S phase. After heat shock the densitometry indicated that there were fewer cells with very high levels, the majority of cells expressed intermediate levels. Perhaps the heat shock had caused the cells to stop at a similar point in the cell cycle. As the cells then recovered from the heat shock the numbers of cells with high levels of topoisomerase II increased. This could be the result of the cells becoming partially synchronised at a particular point in the cell cycle. Alternatively the topoisomerase increase may be a stress response preparing for repair of damaged DNA. The reason for the increase might be established by combining the IHC with fluorescent markers that distinguish different stages of the cell cycle. If those cells that have high levels of topoisomerase II are all at the same stage in the cell cycle, this would suggest there is a synchronisation occurring.

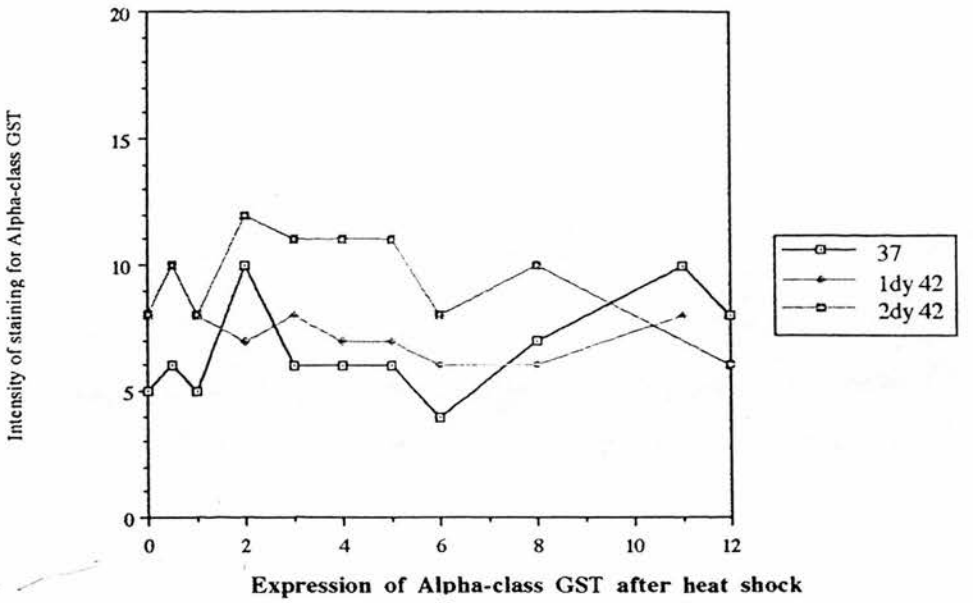
III.2. Further investigation of expression of GST in heat shocked NCI H322 cells

As Pi and Mu class GST showed increased staining after a 5 day recovery period, I felt it useful to repeat the heat shock with longer recovery times to establish when the levels returned to normal. A more comprehensive experiment was carried out. Several time points were taken while the cells were at 42°C and daily time points were taken during the recovery period for up to 10 days following the heat shock. Control time points at 37°C were also taken daily throughout the experiment.

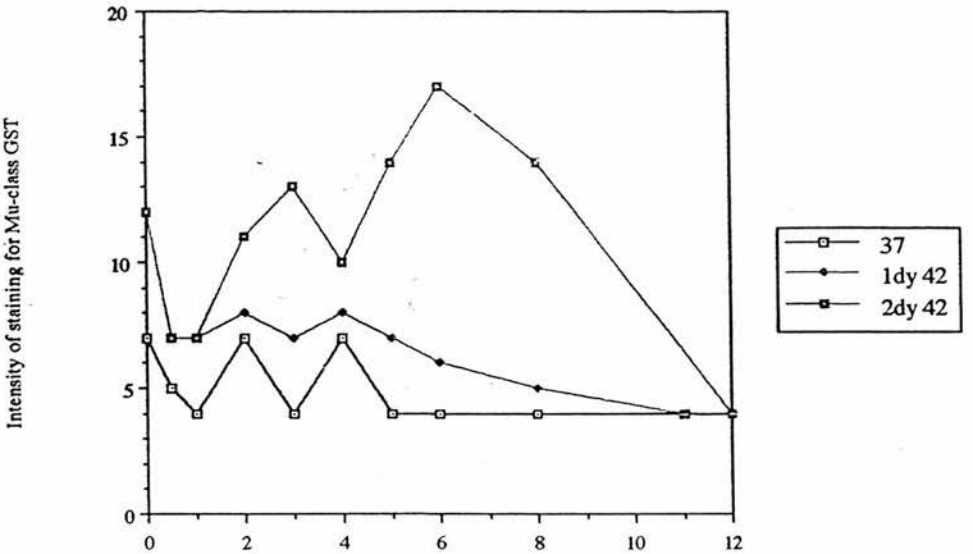
Figure 4: Glutathione S-transferase levels in heat shocked NCI H322 cells up to day 10 recovery.

10^5 NCI H322 cells were seeded in 9 cm^2 slide flasks they were then exposed to 42°C for 12, 24 or 48 hours. Cells were also exposed to 42°C for either 24 (1d 42) or 48 hours (2d 42) and then returned to 37°C for up to ten days. Control cells were left at 37°C for the duration of the experiment. Cells were fixed after each 24 hour period. Day 0 is when cells were initially placed at 42°C, hence transition from 42°C to 37°C occurred on either day 1 (1d 42) or day 2 (2d 42). Cells were fixed in 50% methanol, 50% acetone. Immunohistochemical analysis was carried out using antibodies to the three major human glutathione S-transferase isoenzymes (GST-A1, GST-M1, GST-P1). Immunohistochemical analysis was carried out as described in chapter II (p40). Staining was visualised using a second antibody linked to horse radish peroxidase and its substrate diaminobenzidine). Staining with GST-A1 and GST-P1 was located in the cytoplasm whereas GST-M1 staining was nuclear. The results were scored blind three times, each time in a different order on a score of 1-6. Results for each time point were added and displayed graphically.

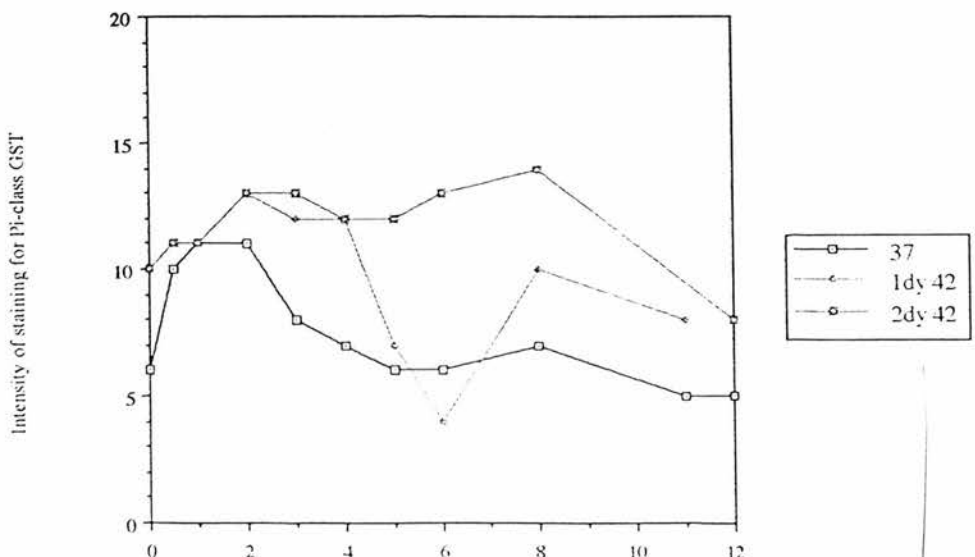
Expression of Glutathione S-transferase following heat shock.



Expression of Alpha-class GST after heat shock



Expression of Mu-class GST after heat shock



Expression of Pi-class GST after heat shock

In these experiments the results were scored blind. To reduce bias each slide was allocated a number at random and each was scored in a different order three times. The results were then tabulated and are shown in graphic form in figure 4. Some of the cell samples appeared to have fairly high levels of GST expression for the first few days after seeding on the slide flasks, although there was variation in the three individual scorings of these samples. This may be due to the artefact of the cell morphology when first plated out. The cells can remain quite rounded when first plated out, making scoring under light microscopy quite difficult.

As in the first IHC experiment the Alpha class GST showed little change throughout the heat shock and recovery periods. There was a small increase seen after 2 days heat shock. After 1 day heat shock the levels did not appear to change at all. One point of interest was that with increasing time growing at 37°C, some cells within the control flasks appear to express much higher levels of Alpha-class GST. This heterogeneity was not seen in the heat shocked or less confluent cells and is investigated further in chapter V.

After one day at 42°C the Mu-class GST expression did not change. After a second day at 42°C and during the first day of recovery, the Mu class GST expression in the nucleus increased before falling slightly and then increased again to peak at day 4 and had fully returned to control cell levels by day 10 recovery. This is consistent with what was noted in the earlier heat shock IHC experiment where levels were found to be increased on days 1 and 5 after the heat shock, but not on day 3.

The Pi-class GST expression increased above control levels after 1 day at 42°C before falling to control levels within 4 days. The levels then

increased again 7 days after the heat shock. Following 2 days at 42°C the level of Pi class GST remains above that of the control cells. By 10 days after the heat shock they had dropped towards the control levels. This would appear to be a slightly different temporal pattern than demonstrated in the first heat shock experiment, where the temporal pattern was similar to that of the 1 day heat shock. The control cells showed heterogeneity in the expression of the Pi class GST with small groups of cells showing higher levels of staining in the confluent cultures (figure 5).

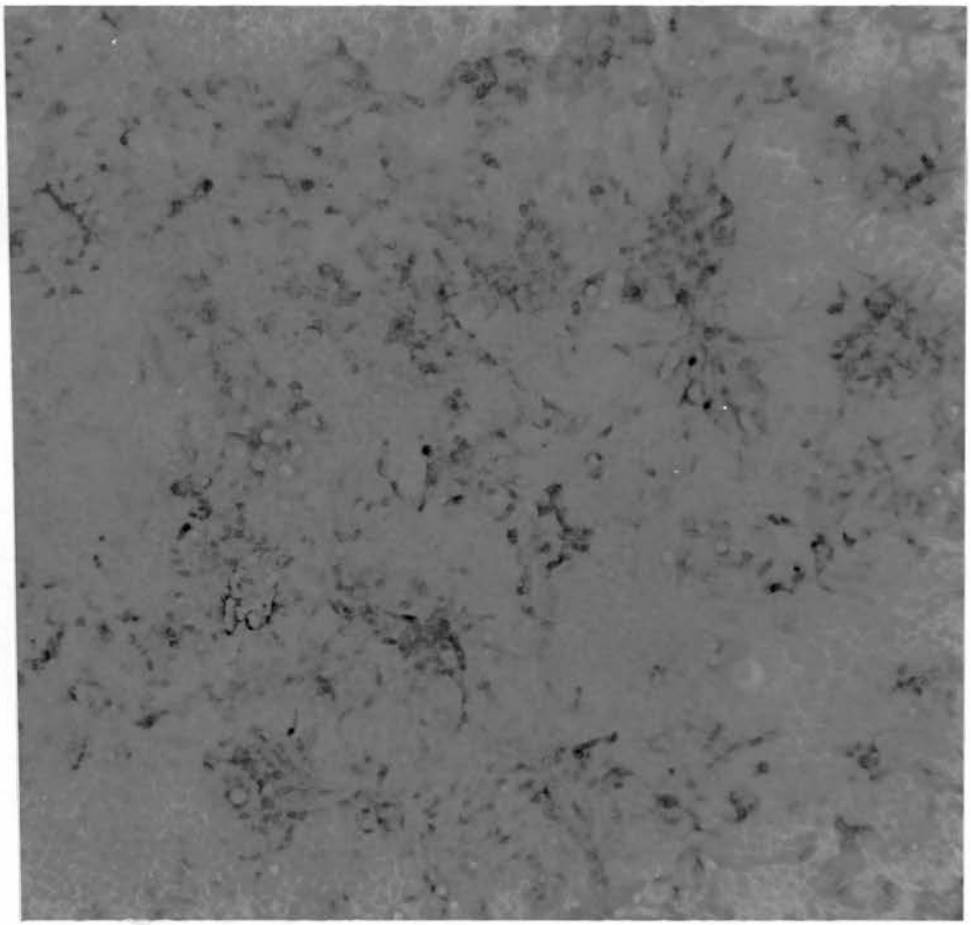
III.3. Summary and discussion

To summarise, various permanent and transient cell line models were analysed for expression of the glutathione S-transferases and other proteins involved in stress response and drug resistance. The major changes noted were as follows. The chlorambucil resistant cell line CHO-Chl^R showed increases in Alpha, Mu and Pi class GSTs. The oxygen resistant cell line, CHO-99 showed decreases in Alpha and Mu class GSTs. The wild type cell line CHO-20 when exposed to high levels of oxygen (CHO-20 (98)) showed staining for Mu class GST within the nucleus. These results are discussed more fully in chapter IV.

Alpha-class GST did not appear to increase substantially with a 2 day 42°C heat shock in NCI H322 cells. However levels in the control cells do appear to increase gradually after about five days. This expression of Alpha and Pi class GST became heterogeneous within the cell population in the confluent cultures; this is investigated further in chapter V. Mu and Pi class GST both show increases following a 2 day 42°C heat shock. This is investigated further in chapter VI.

Figure 5: Heterogeneity of staining for GST in confluent NCI H322 cells.

NCI H322 cells were seeded, heat shocked and then stained for Pi class GST as described in figure 4. The clusters of Pi class staining cells were photographed under light microscopy (100x).



Only one out of the five cytochrome P450 antisera assayed, detected P450 present in the NCI H322 cell line. In preneoplasia the cytochrome P450 show a general decrease in levels, such a decrease was not seen for this P450. Cells in culture tend to have low levels of the P450 as the levels of P450's tend to decrease during the primary culture period from the normal *in vivo* tissue levels. Therefore it is difficult to investigate the relationship between GST induction following stress and the depression of the P450's. There are some cell culture models in which P450's are expressed, either by retaining expression in primary cell cultures or using various induction models. These models may be worthwhile studying.

CHAPTER IV

GST expression in chlorambucil and oxygen resistant CHO cells

IV. GST EXPRESSION IN CHLORAMBUCIL AND OXYGEN RESISTANT CHO CELLS

Elevated γGT and GST levels have been detected in a variety of drug resistant cell lines, as well as in preneoplastic nodules (Ahmad *et al*, 1987; Lewis *et al*, 1988a; Deml and Oesterle, 1980). Mice treated with a non toxic dose of cyclophosphamide were shown to become resistant to a subsequent normally lethal dose of cyclophosphamide (Adams *et al*, 1985). Both Alpha class GST and γGT levels were also shown to be elevated in the livers of these drug primed mice. The chlorambucil resistant cell line (CHO-Chl^R) had been shown to have elevated GST and γGT activity, as well as elevated GSH levels (Lewis *et al*, 1987). This was perceived to be a good model for studying any co-ordinate regulation of GST and γGT with the intention of determining the factors involved in regulation of a co-ordinate response. To this end the regulation of Alpha class GST and γGT in these cells was studied.

Another possible stress in which GSTs may play a role is in oxidative stress. Oxidative stress is known to be caused by various mechanisms such as respiration, phase I drug metabolising enzymes, radiation and a variety of chemicals, including the anthracyclines. Oxygen radicals $O_2\cdot^-$, OH· and H_2O_2 attack unsaturated double bonds in DNA, protein and polyunsaturated lipids causing peroxidation. Kimball *et al* (1976) demonstrated a lung antioxidant response when rat lung tissues were exposed to hyperoxic conditions. They measured the activities of a variety of enzymes and showed superoxide dismutase, glucose-6-phosphate dehydrogenase, glutathione reductase and glutathione peroxidase activities were all increased; as well as levels of reduced glutathione. The Alpha class

GSTs in the lung have been shown to have peroxidase activity towards lipid hydroperoxides and may therefore have an important role in prevention of lipid peroxidation (Singhal *et al*, 1992). A DNA hydroperoxide activity has also been associated with the Mu class GSTs of the rat (Tan *et al*, 1988). Due to the potential role of GST subunits in protecting cells from oxidative stress, the relationship between oxidative stress and the GSTs was investigated. A Chinese hamster ovary cell line selected for resistance to 98% oxygen was obtained from Van der Valk (1985) with the aim of investigating variation in the GST levels. The induction of GST, by transient exposure of the wild type cells to hyperoxic conditions, was also investigated.

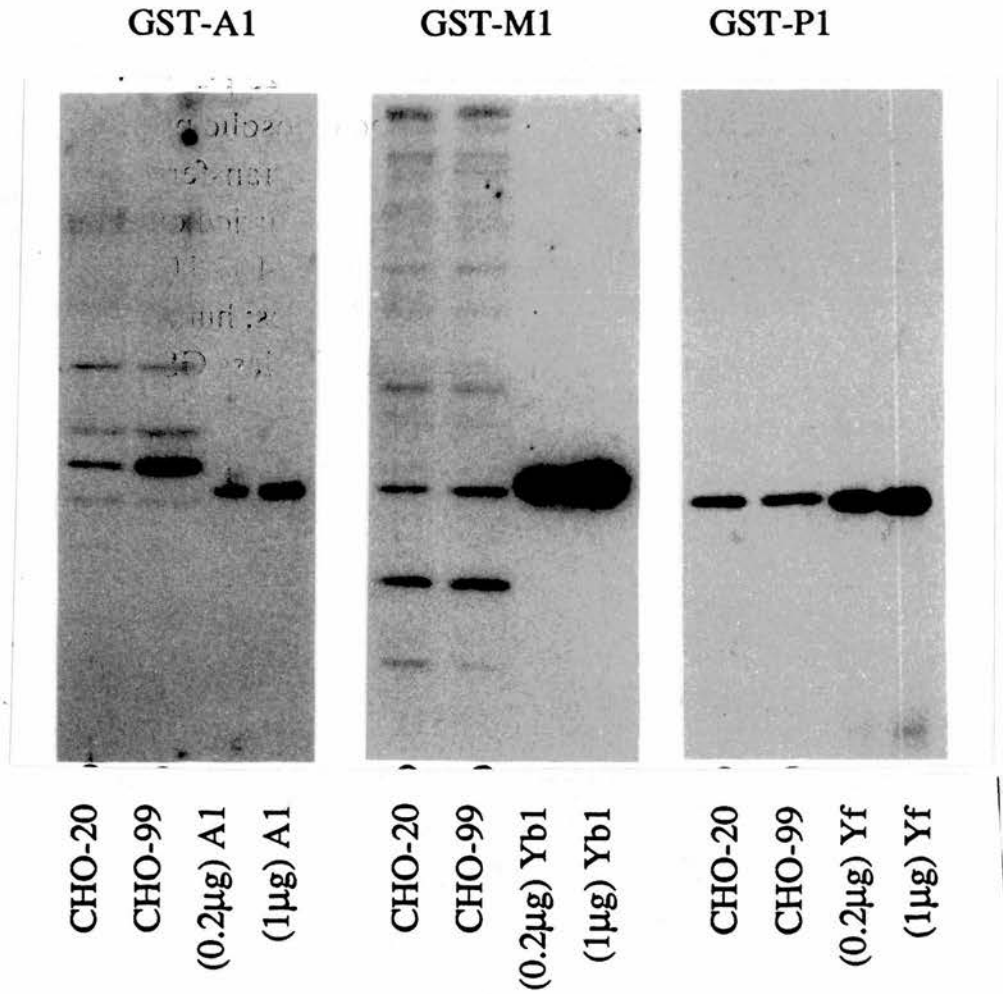
IV.1. Oxygen resistance and transient oxidative stress

A CHO cell line was developed by Van der Valk *et al* (1985) using increasing percentages of an oxygen mixture. The cell line will grow and divide satisfactorily in a mixture of 99% O_2 and 1% CO_2. Immunohistochemical analysis of this oxygen resistant cell line, CHO-99, showed a decrease in Alpha and Mu class GST in the cytosol. However, when the oxygen sensitive wild type cell line, CHO-20, was exposed to high levels of oxygen, it showed staining for Mu-class GST within the nucleus.

To investigate the changes in Alpha class GST, protein was prepared from the cytosols of the oxygen resistant (CHO-99) and oxygen sensitive (CHO-20) cell lines. Western blot analysis showed an increase in one of the Alpha class GSTs in the oxygen resistant cells CHO-99 (figure 6). The molecular weight of this protein was somewhat greater than the human Alpha class GST standard, similar to that of the rat Yc protein. This

Figure 6: GST protein expression in oxygen sensitive and oxygen resistant CHO cell lines.

Cytosolic protein was prepared from the oxygen resistant (CHO-99) and oxygen sensitive (CHO-20) cell lines. 25μg protein was fractionated on SDS-PAGE, transferred to nitrocellulose membrane and Western blot analysis was carried out with antisera raised against human GST-A1, GST-M1 and GST-P1. GST standards were run alongside the samples; human Alpha class GST (A1), mouse Mu class GST (Yb_1) and mouse Pi class GST (Yf).


GST-A1
GST-M1
GST-P1
CHO-20
CHO-99
(0.2µg) A1
(1µg) A1
CHO-20
CHO-99
(0.2µg) Yb1
(1µg) Yb1
CHO-20
CHO-99
(0.2µg) Yf
(1µg) Yf

disagreed with the IHC analysis that suggested Alpha class GST levels were lower in the CHO-99 cells than in the CHO-20 progenitor cell line (table 5). This is difficult to explain except for a technical problem such as loss of protein in this sample. Repetition with the Alpha class GST antisera or staining for other cytosolic proteins may address this discrepancy.

RNA was prepared from both the oxygen resistant (CHO-99) and oxygen sensitive (CHO-20) cell lines. Both cell lines were also exposed to 98% O_2 for two days before RNA was prepared. This exposure was toxic to the wild type CHO-20 cells whereas the CHO-99 cells grew and divided successfully at this exposure. Northern analysis showed the level of Alpha class GST encoding mRNA was increased in the CHO-99 cells. The level of an identical sized mRNA was also induced in the CHO-20 cells on exposure to 98% O_2, 2% CO_2 (figure 7). The CHO-99 cells showed no further increase in Alpha class GST mRNA levels when exposed to 98% O_2.

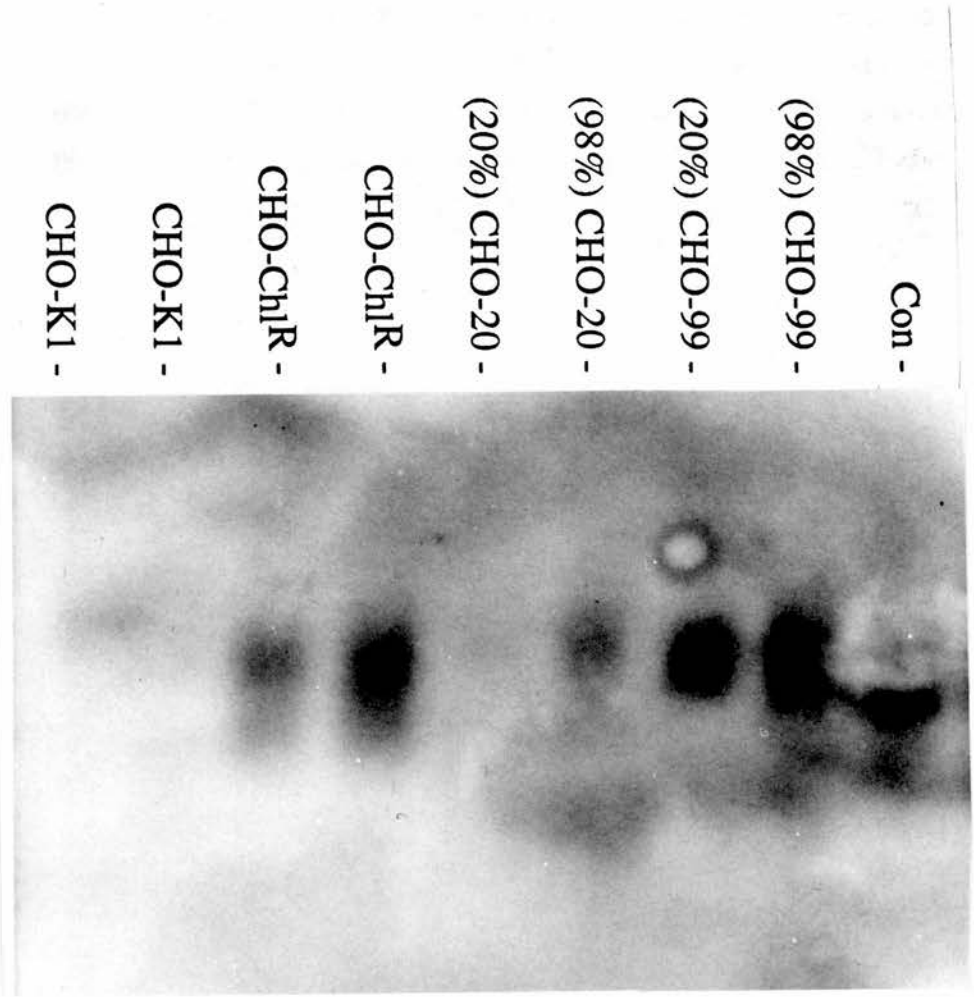
IV.2. Chlorambucil resistance

The chlorambucil resistant cell line CHO-Chl^R showed increases in Alpha, Mu and Pi class GSTs with IHC. This cell line had been shown to have increased activity towards the GST substrate, CDNB, and increased γGT activity (Lewis *et al*, 1988). Increased GST protein was also detected by Western blotting. The major increases were in the Alpha class GSTs where increases in two proteins were seen, one with a molecular weight similar to the human Alpha class GST standard and one with a molecular weight greater than the standard. Immunological data suggest these are analogous to the Ya and Yc subunits of rat.

Figure 7: Alpha class GST mRNA in chlorambucil and oxygen resistant and oxygen treated CHO cell lines.

RNA was prepared using the LiCl method (chapter II) from oxygen sensitive (CHO-20) and oxygen resistant (CHO-99) cell lines. Cells were grown either in 2% CO_2: 98% air (20%) or 2% CO_2: 98% O_2 (98%) for 48 hours before harvesting. RNA was prepared using the LiCl method in duplicate from both chlorambucil sensitive (CHO-K1) and resistant CHO cells (CHO-Chl^R). Samples were then subjected to denaturing agarose gel electrophoresis and transferred to nitrocellulose before being hybridised with a ^{32}P labelled 942 bp fragment of pMP37 (GST-A1). Rat liver RNA was run alongside.

GST-A1


Con -
(98%) CHO-99 -
(20%) CHO-99 -
(98%) CHO-20 -
(20%) CHO-20 -
CHO-ChlR -
CHO-ChlR -
CHO-K1 -
CHO-K1 -

RNA was prepared from the chlorambucil resistant cell line and subjected to Northern analysis using a 942 bp fragment prepared from a cDNA clone of GST-A1, one of the two human Alpha class GSTs. Figure 7 shows the Alpha class GST RNA levels are increased notably in the chlorambucil resistant cells. This mRNA presumably encodes at least one of the two Alpha class GST proteins detected by Lewis *et al* (1988b). The human GST-A1 gene shows 80% similarity at the nucleotide level over the coding region with both the Ya and Yc coding sequences from the rat (table 3). By immunological analysis the hamster proteins are orthologous to the Ya and Yc subunits of rat (A.D.Lewis [1988], PhD thesis). Although it might be expected that the human A1 cDNA would detect both mRNAs their actual homology at the nucleotide level awaits cloning of the genes. Specific probes, however, might be developed to differentiate between the mRNAs encoding the two separate subunits.

To determine the cause of the increased mRNA levels, Southern blot analysis was carried out on the CHO-Chl^R cell line and its progenitor CHO-K1. Figure 8 shows the DNA encoding the Alpha class GSTs was amplified. Southern blot analysis of the γ-glutamyl transpeptidase (γGT) was also carried out on the same blot. Figure 9 shows the DNA encoding the γGT protein is not amplified, therefore it also acts as an internal control for the amount of DNA loaded on the gel. Two fold dilutions of the CHO-Chl^R DNA were run alongside the wild type CHO-K1 DNA. From this the amplification can be estimated to be approximately 4-8 fold (figure 10).

Figure 8: Amplification of DNA encoding Alpha class GST in a chlorambucil resistant cell line.

DNA was prepared in duplicate from separate flasks of chlorambucil resistant (CHO-Chl^R) and sensitive (CHO-K1) cell lines. DNA's were digested with *Taq* I, *Eco* RI, *Pvu* II and *Pst* I and 5μg of each were fractionated on a 1% agarose gel and transferred to Hybond-N membrane. Fragments hybridising to the Alpha class GST were detected using a 942 bp cDNA clone of human GST-A1 (pMP37).

Southern Blot Analysis of CHO-K1 and CHO-Chl^r
Probed with a Human alpha-class cDNA

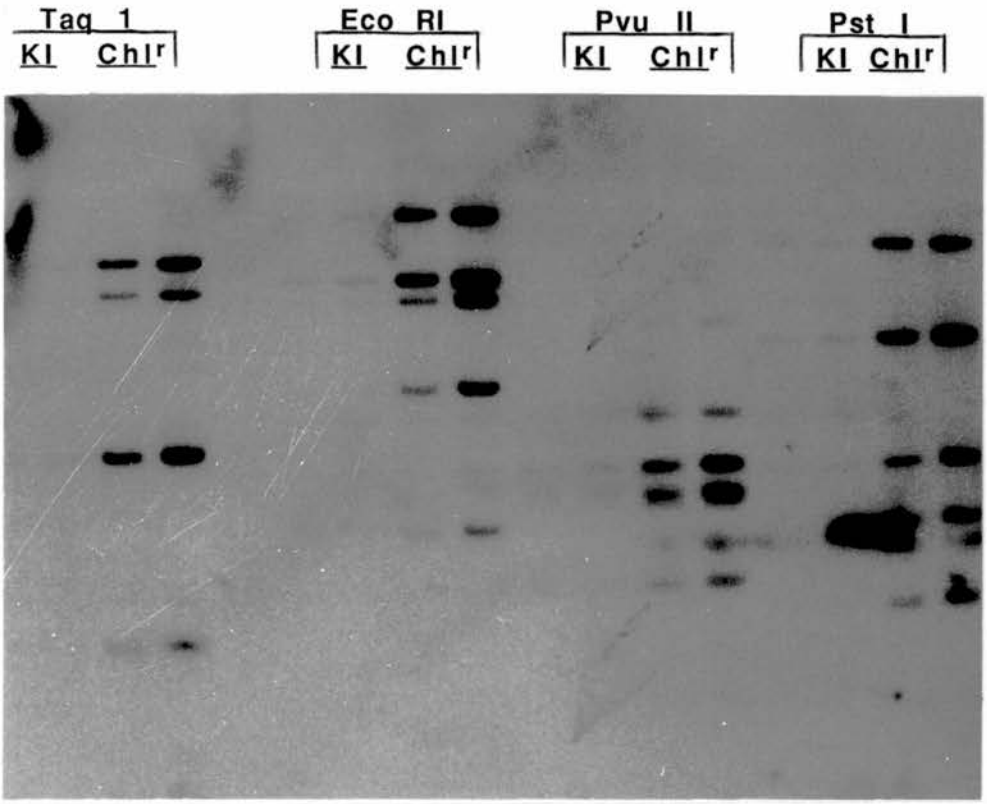


Figure 9: Southern analysis of γGT in a chlorambucil resistant cell line.

The Hybond-N membrane described in figure 8 was stripped of ^{32}P labelled Alpha class GST cDNA, autoradiographed to ensure complete removal of the probe and re-hybridised with a rat kidney γ-glutamyl transpeptidase cDNA fragment.

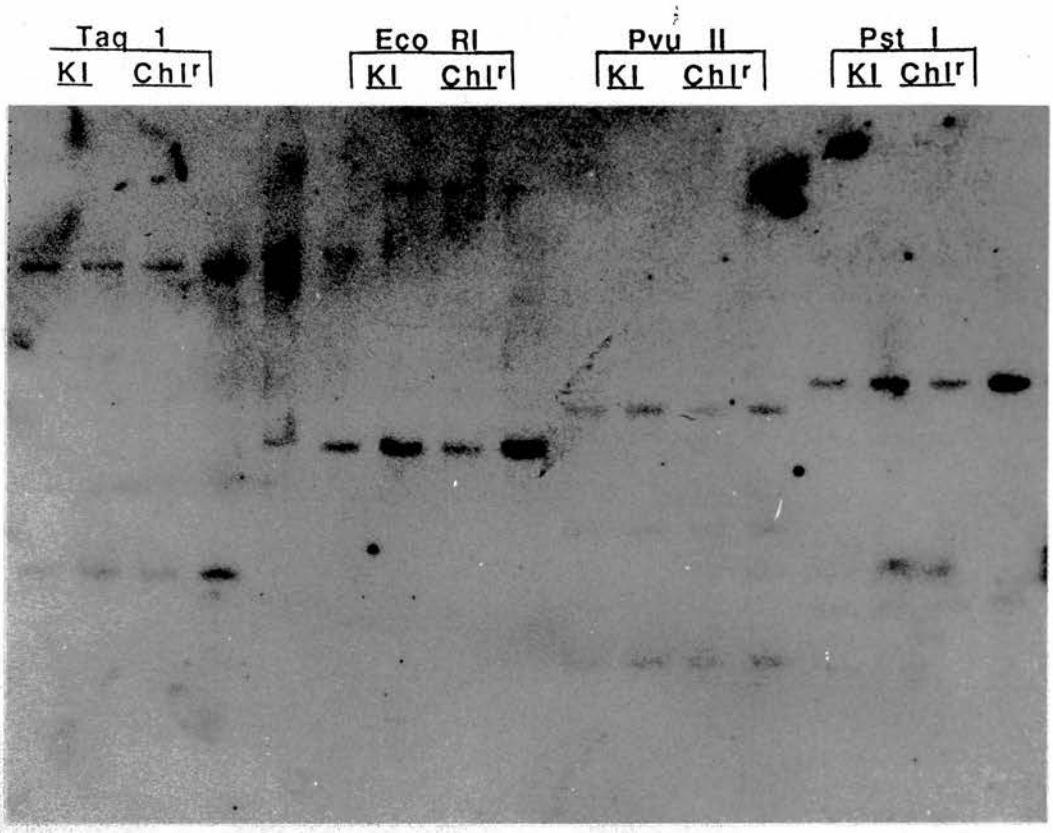

Taq 1
KI Chl^r
Eco RI
KI Chl^r
Pvu II
KI Chl^r
Pst I
KI Chl^r

Figure 10; Estimation of fold amplification of Alpha class GST in a chlorambucil resistant cell line.

DNA from chlorambucil resistant (CHO-Chl^R) and its progenitor cell line (CHO-K1) were digested with *Pst* I. Two fold serial dilutions of the *Pst* I digested CHO-Chl^R DNA were run alongside 5µg of CHO-K1 *Pst* I digested DNA on a 1% agarose gel. DNA's were transferred to Hybond-N and fragments hybridising to the Alpha class GST were detected using a 942 bp cDNA clone of human GST-A1 (pMP37).

- 0.6 µg CHO-ChlR
- 1.2 µg CHO-ChlR
- 2.5 µg CHO-ChlR
- 5 µg CHO-ChlR
- 5 µg CHO-K1

IV.3. Discussion

IV.3.1. Chlorambucil resistance

Robson *et al* (1987) showed the chlorambucil resistant CHO cell line had reduced levels of DNA cross linking activity when treated with the nitrogen mustard, mechlorethamine. They showed that isolated nuclei from the resistant and sensitive cell lines had the same number of cross links and there was no difference in the cross link repair efficiency. This suggested the difference was in the amount of drug reaching the target.

The resistant cells showed increases in activity towards CDNB (3x), ethacrynic acid (2x), cumene hydroperoxide (5x) and showed no activity for styrene oxide. These are general substrates for measuring total GST, Pi class GST, Alpha class GST and Mu class GST respectively implying increases in Pi and especially Alpha class GSTs. IHC and dot blots showed an increase in the levels of Alpha class GST and to a certain extent also Pi class GST (Robson *et al*, 1986).

Alex Lewis had found increased γ-glutamyl transpeptidase (γGT) but not glutathione reductase, γ-glutamyl cysteinyl synthase or selenium dependent glutathione peroxidase activities (Lewis *et al,* 1988). He then showed a dramatic overexpression of the Alpha class GST subunits Yc and Ya using Western blot analysis. Pi and Mu class GST subunits were not shown to be elevated significantly.

Northern and Southern analyses demonstrated that Alpha class GST mRNA was overexpressed in the chlorambucil resistant cells and that the DNA encoding the Alpha class GST was amplified about 4-8 fold in these

cells accounting at least in part for the mRNA increase. The γGT encoding DNA was shown not to be amplified. The Alpha class GST and γGT are not regulated in the same manner suggesting there was no co-ordinate mechanism for regulation.

The fact that an Alpha class GST gene is found to be amplified, and the protein overexpressed, in a chlorambucil resistant cell line, contributes to the mounting body of evidence suggesting the GSTs and more specifically the Alpha class subunits are an important mechanism of resistance to chlorambucil. Inhibitors of GST have been shown to reverse the resistance to chlorambucil (Hall *et al*, 1989). Alpha class GSTs have been shown to be overexpressed in other cell lines resistant to alkylating agents (Buller *et al*, 1987; Evans *et al*, 1987; Schecter *et al*, 1991). Since this work was completed further studies have demonstrated reactive intermediates of alkylating agents including chlorambucil to be GST substrates (Colvin and Hilton, 1988; Bolton *et al*, 1991). Transfection studies have also shown that heterologous expression of human GST-A1 or GST-P1 can lead to resistance to chlorambucil (Black *et al*, 1990; Puchalski and Fahl, 1990). Evidence is accumulating that GSTs are involved in tumours *in vivo* (Schisselbauer *et al*, 1990; Schecter and Alaoui-Jamali, 1991). The amplification of GST in a chlorambucil resistant cell line demonstrates a role for amplification in GST mediated drug resistance.

IV.3.2. Oxygen stress and resistance

The CHO-99 cells were developed through culture in stepwise increases in O_2 (Van der Valk *et al*, 1985). The phenotype is stable with the cells growing well in 99% O_2. Their generation times are 24 and 36 hours

for growth in 20% and 99% O_2 respectively, compared with a 12 hour doubling time for the O_2 sensitive progenitor cell line CHO-20 at 20% O_2. Compared with the CHO-20 cells the CHO-99 cells have an irregular shape; increased mitochondrial volume [1.8 fold]; increased peroxisomal volume [2 fold], as well as increased activity for CuZn superoxide dismutase [2.5 fold]; Mn superoxide dismutase [2.1 fold]; catalase [4 fold] and selenium dependent glutathione peroxidase (GPX) [1.9 fold](Van der Valk *et al*, 1985).

Western and Northern blot analyses showed both, induction of an Alpha class GST subunit with a similar molecular weight as the rat Yc subunit, and an increase in an Alpha class GST mRNA. A similar sized mRNA is induced when the oxygen sensitive cells were exposed to 98% O_2. The oxygen resistant cells, when grown in 98% O_2 do not induce the Alpha class GST mRNA any further, however it is already significantly induced. This indicates the RNA may already be maximally expressed, constitutively in the oxygen resistant cells.

This data showing overexpression of Alpha class GST in an O_2 resistant cell line, as well as the induction of the same GST by oxidative stress strongly suggests a role for this Alpha class GST in oxidative stress. The Alpha class GST of rat has recently been shown to be capable of having a role in preventing lipid peroxidation, a target for oxygen radicals (Singhal *et al*, 1992). Rushmore *et al* (1990, 1991) have demonstrated the inducibility of the rat Ya gene by antioxidants is mediated by an antioxidant response element (ARE) located in the 5' upstream regulatory sequences.

As yet, only two Alpha class GST isoenzymes have been described in Syrian golden or Chinese hamsters (Smith *et al*, 1980; Jenson and

MacKay, 1990). Boogards *et al* (1992) have purified two Alpha class isoenzymes from Syrian golden hamster liver and kidney; these were shown to be the heterodimer, A_1A_2 and the homodimer, A_1A_1. N-terminal sequence established their class but not their relationship to the Alpha class subunits of other species. It is not clear how the proteins examined in the CHO cells correspond to these.

IV.3.3. Link between chlorambucil and oxidative stress

Chlorambucil is an alkylating agent, but its mechanism of toxicity has not been fully established. It is possible that the Yc Alpha class GST subunit in CHO cells may have a similar function in both chlorambucil and oxidative stresses. As the reason for the overexpression of the Alpha class GST is gene amplification, it is possible that only one of the Alpha class subunits is involved in the resistance to chlorambucil. The second highly expressed subunit may be overexpressed due to co-amplification as the subunits are genetically closely linked. The other subunit however may have a role more specifically in the prevention of chlorambucil toxicity. It is of course, entirely conceivable that both subunits have a similar function, hence overexpression of either may lead to the same phenotype. More detailed knowledge of the substrate specificities of the individual subunits is required to enable establishment of their precise role. Some idea may be gained by establishing if the cell lines show cross resistance to both O_2 and chlorambucil.

CHAPTER V

GST's are regulated by a conditioned media response in a lung tumour derived cell line NCI-H322

V. GST'S ARE REGULATED BY A CONDITIONED MEDIA RESPONSE IN A LUNG TUMOUR DERIVED CELL LINE NCI-H322

V.0.1. Role of GST in the lung

The lung is the first line of defence against a variety of toxins inhaled from the environment. The GSTs may play an important role in metabolising a variety of these toxins. In support of this there is some evidence that lack of GST-M1 isoenzyme increases susceptibility to lung cancer, suggesting this isoenzyme plays an important role in detoxification of a carcinogen in cigarette smoke (Seidegard *et al*, 1986, 1990).

The lung is also exposed to high levels of oxidative stress. Although the lung has high levels of various enzymes such as superoxide dismutase and catalase to combat the high levels of superoxide present in the lung, there is still significant damage from free radicals, especially through lipid peroxidation and DNA hydroperoxides. The Alpha class GSTs in the lung have been shown to have peroxidase activity towards lipid hydroperoxides and may therefore have an important role in prevention of lipid peroxidation (Singhal *et al*, 1992).

There are a variety of cell types in the lung which form tumours. The prognosis of the tumour is very dependent on the cell type (Bergsagel and Feld, 1986; Ruckdeschel *et al*, 1986). Small cell carcinomas tend to be initially sensitive to chemotherapy but acquire cross resistance to a variety of drugs readily. The non small cell lung carcinomas comprise a large group of different histological types derived from a variety of cell types, but they do have some clinical similarities, such as a tendency to show intrinsic resistance to a variety of chemicals.

The NCI H322 cell line was derived from an untreated non small cell lung carcinoma (NSCLC), a bronchio-alveolar carcinoma (Falzon *et al*, 1986). These cells demonstrate intrinsic resistance to a variety of drugs including adriamycin and melphalan when compared to an untreated small cell lung carcinoma (SCLC) cell line (Carmichael *et al*, 1988c). This pattern of chemosensitivity, and the levels of GSH and GSH related enzyme activities, (GST, glutathione reductase and γGT) are typical of other NSCLC cell lines and *in vivo* tumours (Carmichael *et al*, 1988b). Since this cell line is lung derived, has had no previous treatment with either chemotherapy or radiotherapy and has been used by others in the lab to study drug resistance, it was felt that this cell line may represent a good model for studying the regulation of the GSTs by stress.

During the immunohistochemical analysis of heat shocked NCI H322 cells, described in Chapter III p66, the levels of Alpha class GST in the control cells gradually increased after five or six days and the cells became heterogeneous for staining for Alpha and Pi class GSTs. It was noticed during Western blot analysis of a CDNB resistant NCI H322 cell subline that the Alpha-class GST levels varied (C.Wareing, personal commun.). The levels between control samples from separate experiments varied significantly, although the differences between the resistant and wild type cell lines always remained consistent. The immunohistochemical analysis suggested that higher levels of GST accumulated as the cells became more confluent. A study, conducted by Alex Lewis, of the GST content of confluent and sub confluent cell lines, including the NCI H322 cells, measured total and selenium dependent glutathione peroxidase activity. This data however was inconclusive of a difference in GSTs between confluent and semi-confluent cells (Alex Lewis, personal

commun.). It did however suggest differences in selenium dependent glutathione peroxidase activity and in reduced GSH.

It was important to establish if any differences in GST levels exist in cells grown under "normal" conditions, and if any differences do exist, what causes these fluctuations. If the expression of GST is found to change while cells are grown under normal conditions, the interpretation of GST changes noted during stress becomes more complicated.

V.1. Western Blot analysis of GST content of NCI H322 cells

To further investigate the source of the fluctuations in the GST levels, Western blot analysis of cytosolic protein samples from a variety of NCI H322 sources was carried out. Samples were randomly selected from control NCI H322 cells grown under normal *in vitro* cell culture conditions. All were grown in RPMI 1640 media with supplements of 10% foetal calf serum and L-glutamine and cultured at 37°C, 5% CO_2, 100% humidity. There were some variations in additives such as HEPES, penicillin and streptomycin. Several different people, C.Wareing, A.Bartoszek and I, prepared the samples using different methods of harvesting and cytosol preparation. The samples were loaded on to a 12% gel, subjected to SDS-PAGE then transferred to nitrocellulose membrane and immunoblotted with antibodies raised against the three major GST isoenzymes from human; A1 [α], M1 [μ] and P1 [π] (figure 11).

Western blot analysis of the Alpha-class GST demonstrated the variation that exists between samples from same cell line grown and prepared under "standard" conditions. The levels vary from an almost undetectable to a strong signal. Pi class GST was present in the cytosols of

Figure 11: Expression of GST protein in control NCI H322 cells

A number of NCI H322 cytosolic protein samples were prepared by A.Bartoszek, C.Wareing and myself. The samples were harvested at different times, following varying protocols. Then, 30μg samples were subjected to SDS-PAGE, transferred to nitrocellulose and Western blotted with Alpha (A1-NM) and Pi (P1-λ) class GST antisera. Human liver (lv), human lung (lg) and GST standards, human Alpha class GST (A1), and mouse Pi class GST (Yf), were run alongside the samples.

- A1 (20ng)
- A1 (4ng)
- lg. (6 µg)
- lv. (0.3 µg)
- 12
- 11
- 10
- 9
- 8
- 7
- 6
- 5
- 4
- 3
- 2
- 1
A1-NM
- Yf (0.05 µg)
- Yf (0.01 µg)
- lg. (6 µg)
- lv. (0.3 µg)
- 12
- 11
- 10
- 9
- 8
- 7
- 6
- 5
- 4
- 3
- 2
- 1
P1-λ

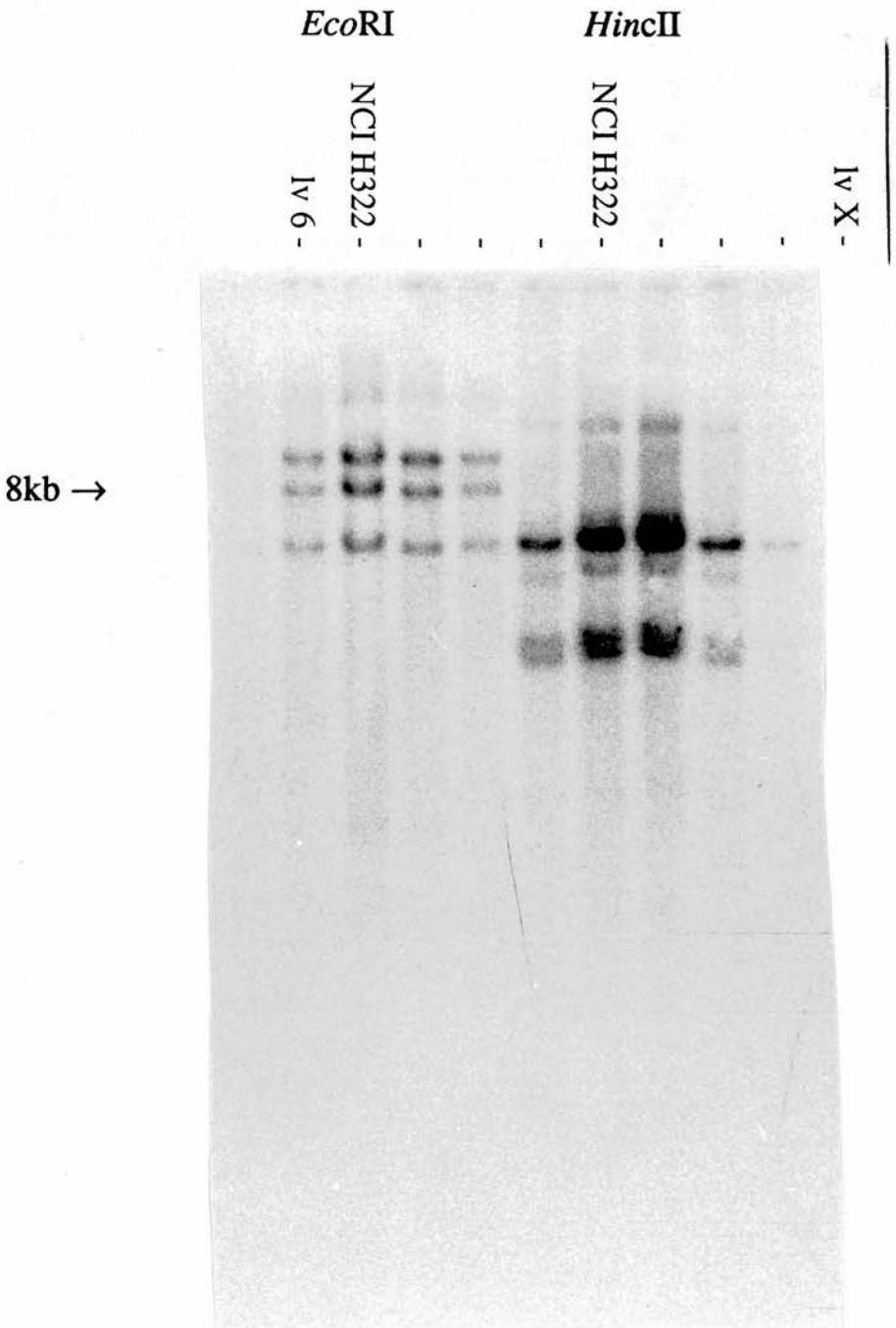
all the NCI H322 cell samples. The amount of Pi class GST varied in a similar manner to the Alpha class GST although the variation was not as great. There was a tendency for Alpha and Pi class GST levels to be lower in the same samples, but no absolute relationship is clear from these samples.

GST-M1 was not detectable in these cells, in either cytosol or nuclei, with antisera raised against the hepatic Mu class GST-M1 confirming observations by others (C.Wareing, A.Lewis, A.Bartoszek, personal commun.). With the Mu class antiserum M1-SL, however, a protein was detected in some samples on long exposures that ran with a similar mobility to the non polymorphic liver band, thought to be GST-M2 (GST-4). This protein also varied in a similar manner to the Alpha and Pi class GSTs. Immunohistochemical analysis had suggested there were low levels of a Mu class GST that was present in the nucleus of non-stressed cells. It may be that the antibodies used, which were raised against the human liver Mu class GST-M1 (μ), do not recognise the lung Mu-class enzymes on a western blot. This will be discussed in greater detail in later chapters.

At this stage, it was important to establish whether the cells used are genotypically null for GST-M1 since approximately 50-55% of the human population have a GST-M1 null phenotype and are homozygous for the allele, GST-M1*0 (Seidegard et al, 1988). This allele is a gene deletion detectable as a restriction fragment length polymorphism (Board, 1981; Seidegard *et al*, 1988). DNA was extracted from NCI H322 cells and digested with *Eco*R I and *Hinc* II and electrophoretically fractionated on a 1% agarose gel and subsequently transferred to Hybond-N. A ^{32}P labelled 400 bp fragment from GST-M1 was used to probe the Southern blot (figure

Figure 12: Southern blot analysis of GST-M1 in NCI H322 cells

Genomic DNA was prepared from NCI H322 cells as described in chapter II, pg 44. DNA digested with the DNA restriction enzymes *Eco* RI and *Hinc* II was fractionated on a 0.8% agarose gel and transferred to Hybond-N before Southern analysis with a ^{32}P labelled 400 bp fragment corresponding to a Mu class GST genomic sequence from J. Taylor (jt14). Marker is 1 kb marker from BRL (M).


EcoRI
HincII
lv 6 -
NCI H322 -
-
-
-
NCI H322 -
-
-
-
lv X -
8kb →

12). Detection of the 8 kb *Eco* RI fragment demonstrated the presence of the GST-M1 gene and therefore the gene deficiency does not explain the lack of GST-M1 expression. The lack of expression is likely to be due to the specific regulation of the gene.

V.2. Effect of time in culture on Alpha and Pi class GSTs

To establish if the variation in the level of expression of the GST subunits was related to the increasing confluence of cells in culture, NCI H322 cells were seeded and fed every day for 13 days. By this time the cells were highly confluent although still looked healthy due to the frequent feeding. Samples were taken every day for 13 days. The cells were harvested and whole cell protein prepared. Western blot analysis with antisera raised against Alpha and Pi class GST show the levels of both are induced approximately 20-50 fold (figure 13).

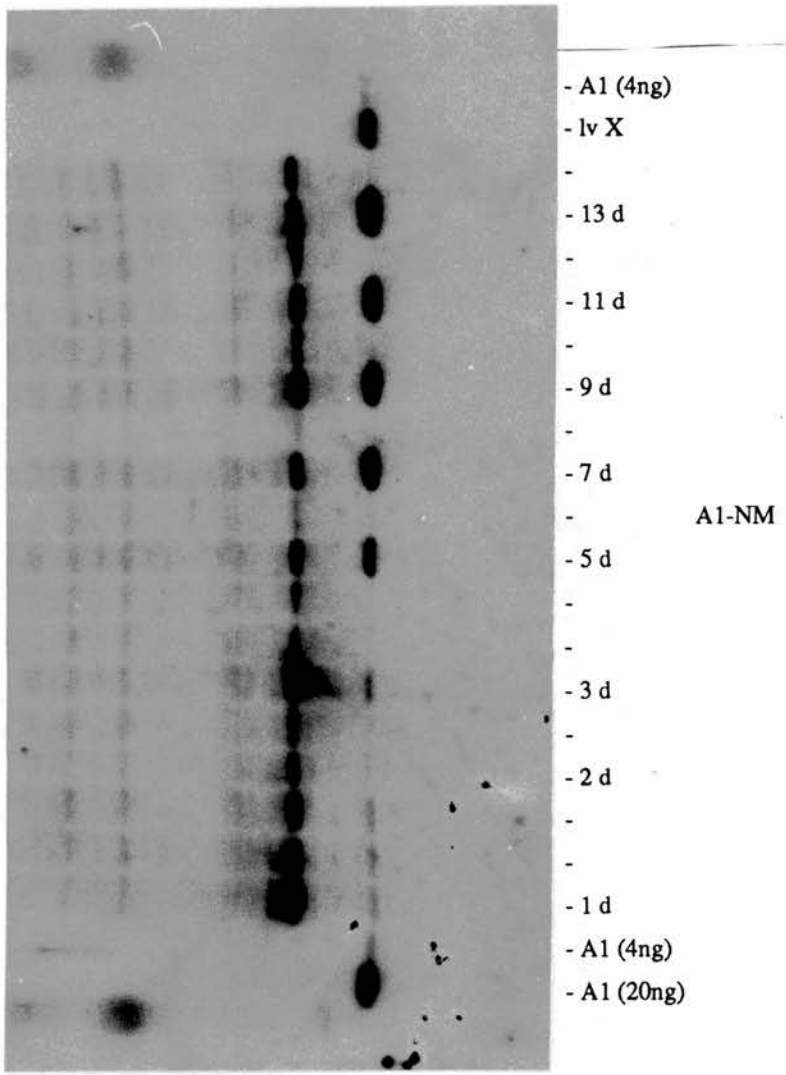
V.3. Effect of culture conditions on the GST content of NCI H322 cells

It was necessary to establish if the variation in GST between the control samples from different experiments was due to differing protein preparations, growth conditions or culture techniques. Cells tend to be fed at different intervals. Also the cells are harvested at different densities, principally because confluence does not inhibit their growth, and the cells continue to grow at high densities.

Cells were plated out at three different densities on four different days using 40 ml of media. These cells were then fed using one of the following three protocols shown in table 6; 1) fed every day, 2) fed alternate days and 3) not fed during the experiment. On the final day the cells were

Figure 13: Induction of Pi and Alpha class GSTs during culture of NCI H322 cells.

$1x10^7$ NCI H322 cells were seeded in 175 cm^2 flasks in 40 ml RMPI + HEPES. Cells were harvested at 1 day intervals over a period of 13 days. The cells were fed every day with 40ml fresh media. Cells were harvested and cytosolic protein was prepared. 25µg samples were subjected to SDS-PAGE, transferred to nitro-cellulose membrane and Western blotted with Alpha and Pi class GST antisera. Human liver (lv) and GST standards, human Alpha class GST (A1) and mouse Pi class GST (Yf), were run alongside the samples. Unlabelled tracks are not relevant to this figure.


- A1 (4ng)
- lv X
-
- 13 d
-
- 11 d
-
- 9 d
-
- 7 d
-
- 5 d
-
-
- 3 d
-
- 2 d
-
-
- 1 d
- A1 (4ng)
- A1 (20ng)
A1-NM

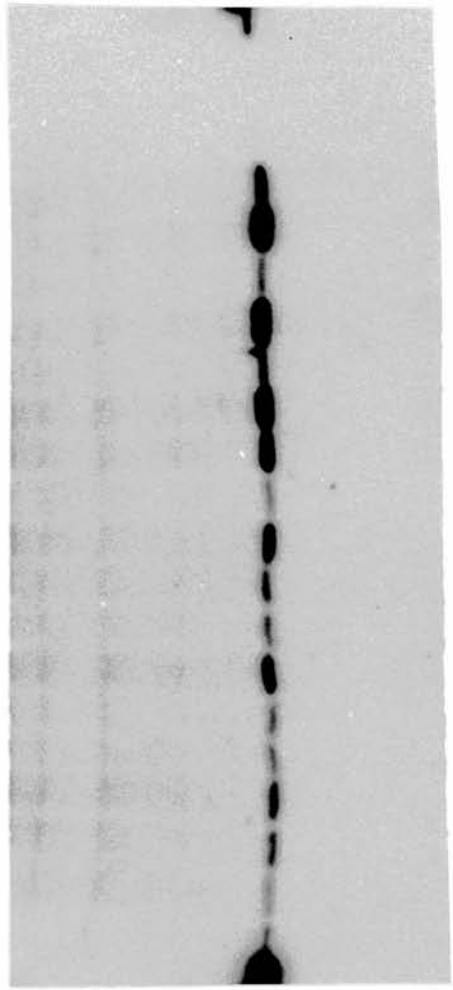

- Yf (20ng)
- lv
- lv
-
- 13 d
-
- 11 d
-
- 9 d
- 7 d
-
- 5 d
-
-
- 3 d
-
- 2 d
-
-
- 1 d
- Yf (20ng)
- Yf (100ng)
P1-λ

Table 6: Protocol for testing effect of feeding and density on GST expression (figure 15).

Cells were plated out at three different densities on 4 different days using 40 ml media. These cells were fed using one of the following three protocols; 1) fed every day, 2) fed alternate days and 3) not fed during the experiment.

No. days in culture		No. cells seeded	Day 1	Day 2	Day 3	Day 4	Day 5
1 day	Fed 1	4×10^7				Seeded	Harvested
2 day	Fed 1	4×10^7			Seeded		Harvested
		1×10^7					
3 day	Fed 3	4×10^7		Seeded	Fed	Fed	Harvested
		1×10^7					
3 day	Fed 1	4×10^7		Seeded			Harvested
		1×10^7					
4 day	Fed 4	4×10^7	Seeded	Fed	Fed	Fed	Harvested
		1×10^7					
		3×10^6					
4 day	Fed 2	4×10^7	Seeded		Fed		Harvested
		1×10^7					
		3×10^6					
4 day	Fed 1	4×10^7	Seeded				Harvested
		1×10^7					
		3×10^6					

photographed before harvesting using trypsin (figure 14). The cells were then fractionated and protein from the cell cytosols prepared. The cytosolic protein samples were then analysed by SDS-PAGE and Western blotted with antisera raised against Alpha, Mu and Pi class GSTs (figure 15).

Alpha- and Pi-class GSTs have a very similar pattern of expression. They are both found at higher levels with increasing density of plating, increasing time in culture and a low frequency of feeding.

The Western blot analysis with one of the GST-M1 antisera, M1-JN, shows a protein in the cytosol with a faster mobility than the major hepatic Mu class GST-M1. The level of this Mu class GST is elevated in the same manner as the Alpha and Pi class GSTs; suggesting this is not a background band. The level of staining for this protein is low indicating that either the protein is at a low abundance or that the antiserum does not cross react well with this GST subunit. The latter is thought likely as several different antibodies raised against hepatic GST-M1 were used and only one detected the band with any clarity, although two others did detect it above background (M1-SL and M1-SP). This suggests the detected protein shares a few antigenic sites with GST-M1, and is therefore likely to belong to the Mu GST class.

The number of Mu class GSTs identified in the last few years has been rapidly increasing and Mu is becoming the largest of the GST classes. John Hayes has antisera raised against rat Yo, Yn, Yk and the rat Theta class isoenzymes GST-E (GST-5 and -12) as well as the major hepatic GST subunits of both human and rat. I have tried all these GST antisera but none detected the protein.

Figure 14: NCI H322 cells before harvesting for figure 15.

NCI H322 cells fed as described in table 6 were washed in PBS and photographed shortly before harvesting for Western blot analysis (figure 15). Cells were photographed under phase contrast light microscopy (100X). $4x10^7$ (h), $1x10^7$ (m) or $3x10^6$ (l) NCI H322 cells were seeded on four subsequent days and harvested on the fifth day. Cells were fed following the protocol in table 6; every day (4d fed 4, 3d fed 3) every alternate day (4d fed 2) or kept in the same media (4d fed 1, 3d fed 1, 2d fed 1, 1d fed 1).

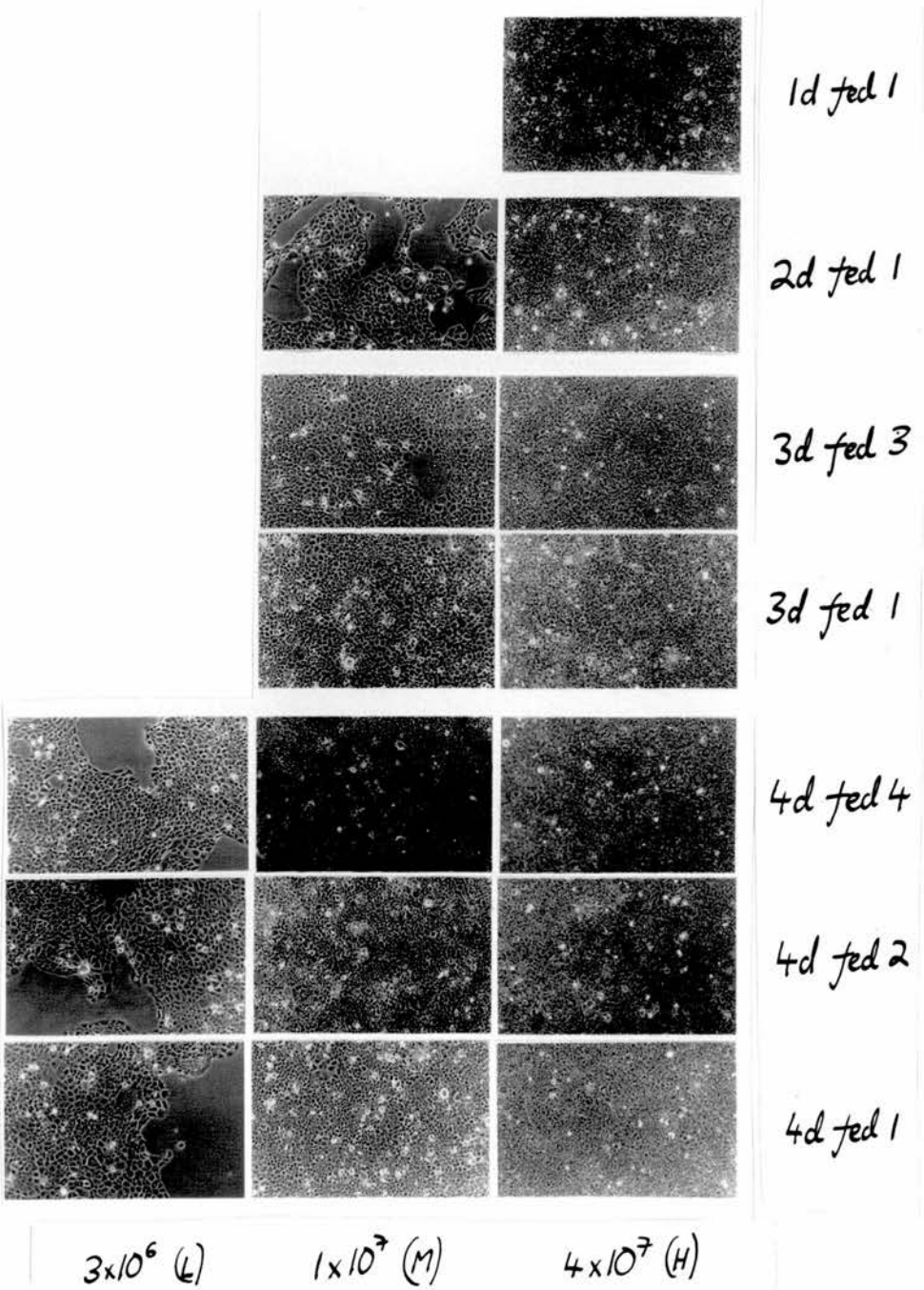
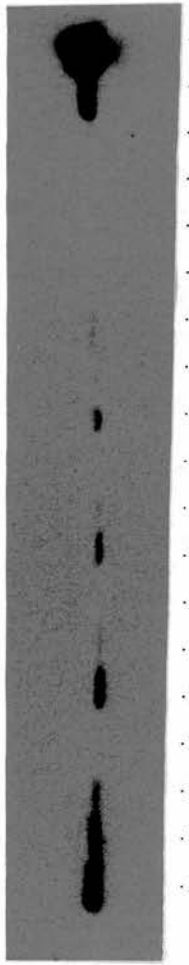

1d fed 1
2d fed 1
3d fed 3
3d fed 1
4d fed 4
4d fed 2
4d fed 1
3x10^6 (L)
1x10^7 (M)
4x10^7 (H)

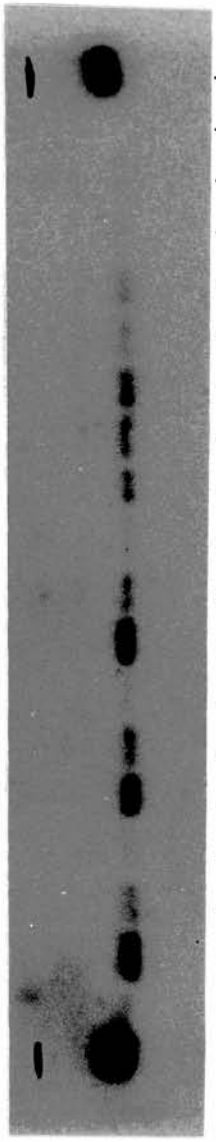
Figure 15: Effect of density and feeding of NCI H322 cells on expression of GSTs.

$4x10^7$ (h), $1x10^7$ (m) or $3x10^6$ (l) NCI H322 cells were seeded on four subsequent days and harvested on the fifth; i.e. cells were in culture for 1, 2, 3 or 4 days (1d, 2d, 3d or 4d). Cells were fed following the protocol in table 6; every day (4d fed 4, 3d fed 3) every alternate day (4d fed 2) or kept in the same media (4d fed 1, 3d fed 1, 2d fed 1, 1d fed 1). Cells were photographed (figure 14), harvested and cytosolic protein was prepared. 20μg samples were subjected to SDS-PAGE, transferred to nitro-cellulose membrane and Western blotted with Alpha, Mu (M1-JN) and Pi class GST antisera. Human liver (lv) and GST standards, human Alpha class GST (A1), mouse Mu class GST (Yb_1) and mouse Pi class GST (Yf), were run alongside the samples. BRL Rainbow markers were loaded on the end tracks as size markers



- lv
- A1
- h fed 1 1d
- m
- h fed 1 2d
- m
- h fed 3 3d
- m
- h fed 1 3d A1-NM
- l
- m fed 4 4d
- h
- l
- m fed 2 4d
- h
- l -
- m fed 1 4d
- h
- A1

- Yb
- Yb
- h fed 1 1d
- m
- h fed 1 2d
- m
- h fed 3 3d
- m
- h fed 1 3d M1-JN
- l
- m fed 4 4d
- h
- l
- m fed 2 4d
- h
- l -
- m fed 1 4d
- h



- Yf
- lv
- h fed 1 1d
- m
- h fed 1 2d
- m
- h fed 3 3d
- m
- h fed 1 3d
- l P1-λ
- m fed 4 4d
- h
- l
- m fed 2 4d
- h
- l -
- m fed 1 4d
- h
- Yf
- Yf

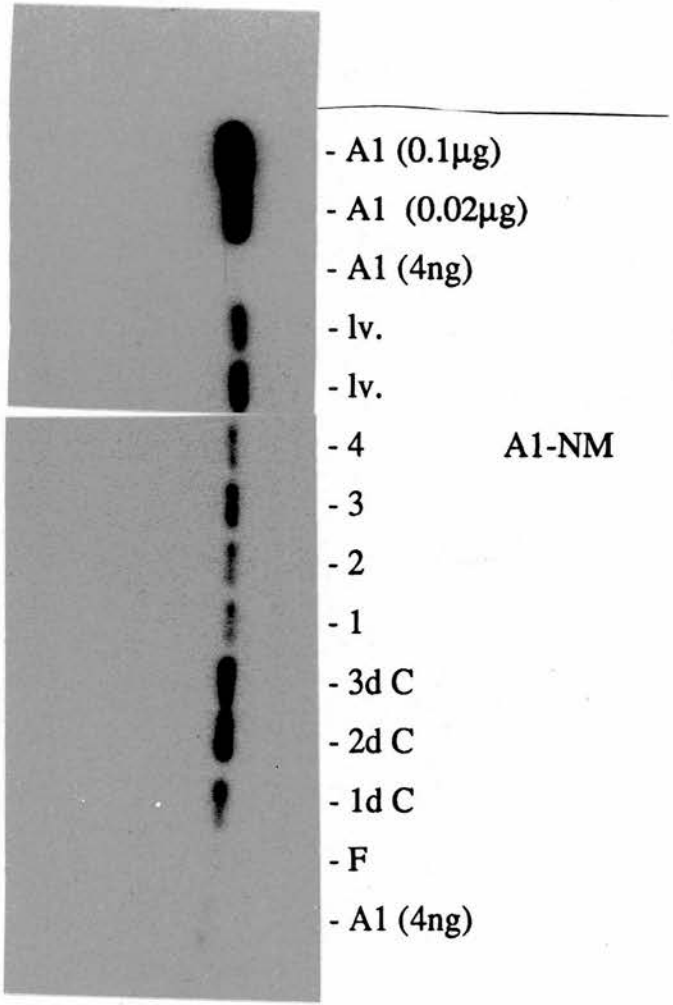
V.4. Induction of GST is mediated by the culture media

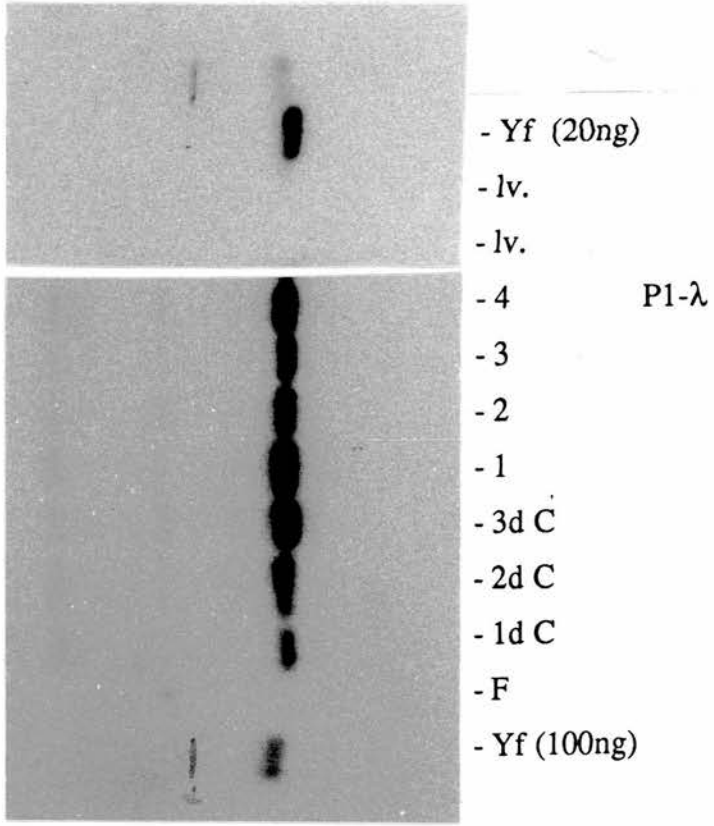
Levels of all three major cytosolic GST classes increase in cells allowed to grow to high density and when infrequently fed. At this stage it is impossible to say if there is more than one factor influencing the GST levels. However the frequency of feeding alters the GST levels; suggesting the effect detected is probably not directly related to the cell density of the cultures. I hypothesised the change in GST levels was a conditioning effect the cells had on the surrounding media. This effect would be intensified by both infrequent changes of media and having increasing numbers of cells in the flask as in cultures grown to high densities. To test this possibility; media from flasks of confluent cells were added to flasks of freshly plated cells. After two days the cells were harvested and analysed for Alpha and Pi class GST expression (figure 16). As predicted, Alpha and Pi class GST levels were increased, suggesting the effect may be mediated by a diffusible factor in the culture medium.

The change in GST levels appears to be related to cell density with the response being mediated through the conditioned culture medium. The induction could be mediated either by the presence of a factor secreted into the media or due to the deficiency of a required nutrient or other molecule. Alternatively, a change such as an alteration in the pH or in the redox state of the media, may mediate the response. The degree of acidity in the media can be correlated with both the cell density, and the frequency of changing the medium. As these cells have the property of rapidly affecting the pH of the media, this latter possibility was tested. The cells were plated out in media in which the pH was altered by adding either HEPES or MOPS buffered solutions (figure 16). The changes in the final pH in this

Figure 16: Effect of conditioning media and varying pH on expression of GST.

NCI H322 cells were seeded at $2x10^7$ and grown for 2 days in 50ml fresh media (F) or in 50ml media conditioned on NCI H322 cells for 1, 2 or 3 days (1d C, 2d C, 3d C). Media was filtered through a Dynaguard syringe filter to ensure no cell carryover. Cells were also seeded and grown for two days in media with the pH altered using HEPES or MOPS. The final pH of the media was as follows (1) pH 7.11 (2) pH 7.13 (3) pH 6.91 (4) pH 7.28. Cells were harvested and cytosolic protein prepared as described in chapter II. 20 μg protein was subjected to Western blot analysis for Alpha and Pi class GST using antisera A1-NM and P1-λ. Human liver (lv) and GST standards (S), human Alpha class GST (A1) and mouse Pi class GST (Yf) were run alongside the samples.


- A1 (0.1µg)
- A1 (0.02µg)
- A1 (4ng)
- lv.
- lv.
- 4
- 3
- 2
- 1
- 3d C
- 2d C
- 1d C
- F
- A1 (4ng)
A1-NM


- Yf (20ng)
- lv.
- lv.
- 4
- 3
- 2
- 1
- 3d C
- 2d C
- 1d C
- F
- Yf (100ng)
P1-λ

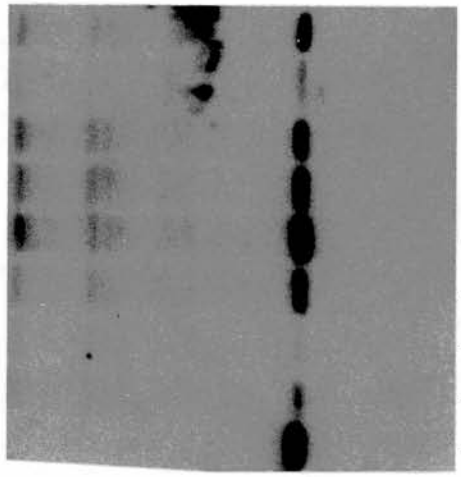
experiment spanned the normal pH range observed in the media from these cells (normally within pH 7 - pH 7.2). However only small changes in Alpha class GST were noted. These were not considered sufficient to cause the observed changes.

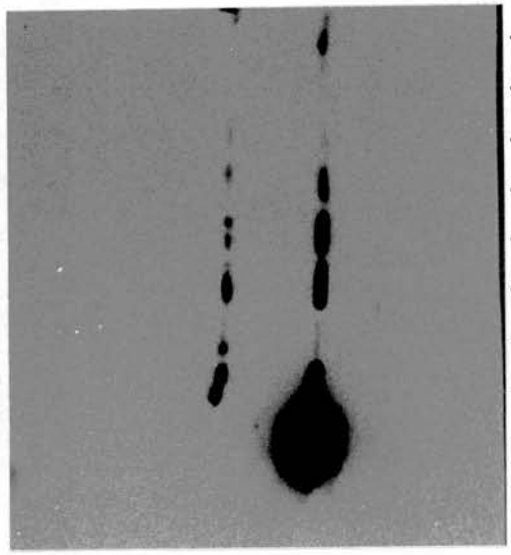
To establish an approximate size for the factor, cells were plated out in conditioned media that had previously been dialysed against fresh media (figure 17). Dialysis was performed with membrane that had a molecular weight cut off of 10-14 kD, enabling the size of the factor causing the GST induction to be approximated. The dialysed media had a dramatically reduced capacity to induce both Alpha and Pi class GST levels, suggesting the inducing factor has a maximum molecular weight of 10-14 kD.

The conditioned media was mixed with fresh media, in various proportions, before the cells were plated out (figure 17). This resulted in an approximately linear response with GST levels being increased in a proportionate fashion to the amount of conditioned media present. If the response was due to the depletion of a required factor or nutrient, one would expect to see a threshold limit, below which no response is seen. However, cells cultured on a mixture containing only 25% conditioned media and 75% fresh media showed an increase in Pi class GST levels. It would be unlikely that this mixture becomes depleted of a required factor or nutrient significantly faster than 100% fresh media. These data are complicated by the observation that the Pi class GST levels in the cells grown in 100% conditioned media were lower than those of the cells grown in 75% conditioned media. The reason for this is not clear but perhaps this response may result from the depletion of a required factor from the media. The Alpha class GST levels did not show this later response so this is

Figure 17: Effect of dialysis and proportion of conditioning and fresh media on the ability to alter GST expression.

$4x10^7$ NCI H322 cells were seeded and grown for 2 days in conditioned media (C) from NCI H322 cells [conditioning cells (Con) were seeded at $2x10^7$ and grown for 4 days without a change of media] mixed with fresh media (F) in varying proportions. Cells were also seeded in conditioned media that had either been dialysed against two changes of fresh media for 3.25 hours (D) or had 10% foetal calf serum added (FCS). Media was filtered through a Dynaguard syringe filter to ensure no cell carryover. Human liver (lv) and GST standards (S), human Alpha class GST (A1) and mouse Pi class GST (Yf) were run alongside the samples.


- C +FCS
- D
- 25% C
- 50% C
- 75% C
- 100% C
- 100% F
- Con
-Yf (100ng)
P1-λ


- C +FCS
- D
- 25% C
- 50% C
- 75% C
- 100% C
- 100% F
- Con
- A1 (100ng)
A1-NM

thought to involve a separate effect from the conditioned media response.

An alternative explanation for the conditioned media response is that the cells are affecting the redox state of the media. Various small molecules that might affect the redox or glutathione status of the media were added as supplements to both fresh and conditioned media. As figure 18 shows, none of the additives have any dramatic effect, on the levels of Alpha or Pi class GSTs, when added to either the fresh or conditioned media. There is some evidence for enhancement of the response. With the addition of dithiothreitol (DTT), buthionine sulphoxamine (BSO) or cysteine some very marginal increases are seen, this experiment was not repeated however. The changes when the additives are added to fresh media are not sufficient to elicit the response noted with the conditioned media.

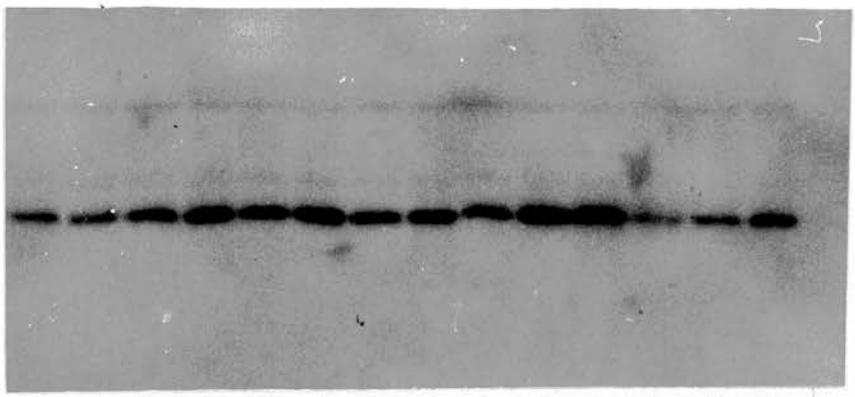
V.5. Regulation of GST at the molecular level

To investigate the nature of the regulation, advantage was taken of a transfected cell line developed in the lab (S. Black). This is a stable transfectant of the human GST-A1 cDNA expressed from a heterologous promoter in MCF-7 cells (139-6). These were plated out in media conditioned as before on the NCI H322 cells. As can be seen from figure 19, growing the transfected cells on conditioned media increased the expression of the transfected GST-A1 in the 139-6 subline of MCF-7. The progenitor cell line did not express GST-M1 when grown in either fresh or conditioned media.

Since the Alpha-class GST is being expressed from a heterologous promoter, increased expression can not be due to regulation by its normal

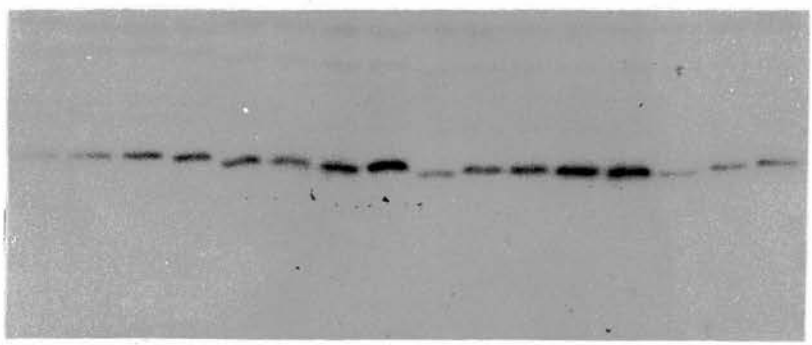
Figure 18: Effect of altering the glutathione and redox state of the media on the NCI H322 conditioning response.

$4x10^7$ NCI H322 cells were seeded in 175 cm^2 flasks. They were grown for 2 days in either 75% fresh (F) or 75% conditioned media (C) mixed with 25% fresh media. Media was filtered through a Dynaguard syringe filter to ensure no cell carryover. Media was conditioned by cells seeded at $2x10^7$ and grown for 4 days in 50 ml RPMI 1640. The following were added to the media; 2.5μg/ml insulin; 1 mM DTT; 1 mM BSO; 5 μg or 50 μg/ml cysteine (Cys); and 1 mM GSH. Cell counts revealed cells grown on conditioned media had ~20% less final cell numbers and cells grown with added insulin had 16% more cells than controls. Cells were photographed, counted, harvested and cytosolic protein was prepared. 20 μg samples were subjected to SDS-PAGE and Western blotted with Alpha and Pi class GST antisera. Human liver (lv) and GST standards (S) were run alongside the samples human Alpha class GST (A1) and mouse Pi class GST (Yf). Note * samples loaded opposite way to others.



- 1 mM GSH (C)
- 1 mM GSH (F)
- 5 µg/ml Cys (F) *
- 5 µg/ml Cys (C) *
- 50 µg/ml Cys (C)
- 50 µg/ml Cys (F)
- (C)
- (F)
- 1 mM BSO (C)
- 1 mM BSO (F)
- 1 mM DTT (C)
- 1 mM DTT (F)
- 2.5 µg/ml insulin (C)
- 2.5 µg/ml insulin (F)

GST-A1

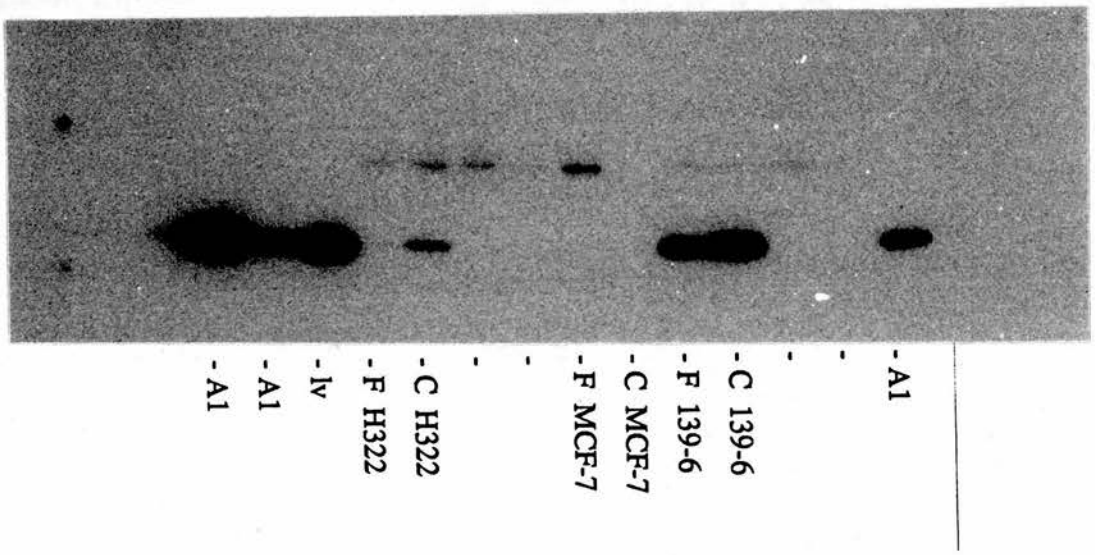


- 1 mM GSH (C)
- 1 mM GSH (F)
- 5 µg/ml Cys (F) *
- 5 µg/ml Cys (C) *
- 50 µg/ml Cys (C)
- 50 µg/ml Cys (F)
- (C)
- (F)
- 1 mM BSO (C)
- 1 mM BSO (F)
- 1 mM DTT (C)
- 1 mM DTT (F)
- 2.5 µg/ml insulin (C)
- 2.5 µg/ml insulin (F)
- lv
- Yf (100ng)

GST-P1

Figure 19; Effect of NCI H322 conditioned media on expression of Alpha class GST from a heterologous promoter.

MCF-7, MCF-7 GST-A1 transfectant 139-6 and NCI H322 cell lines were seeded and grown for 3 days either in fresh RPMI 1640 (-F) or RPMI 1640 conditioned for 4 days on NCI H322 cells (-C). Media was filtered through a Dynaguard syringe filter to ensure no cell carryover. Cell numbers seeded were; $2x10^7$ NCI H322 (H322); $1x10^7$ human breast cancer cell line (MCF-7); $1x10^7$ GST-A1 transfectant derived from MCF-7 cell line (139-6). Cells were harvested and cytosolic protein was prepared. 20µg samples were subjected to SDS-PAGE, transferred to nitro-cellulose membrane and Western blotted with Alpha class GST antisera (A1-NM). Human liver (lv) and the GST standard, human Alpha class GST (A1), were run alongside the samples.


- A1
-
-
- C 139-6
- F 139-6
- C MCF-7
- F MCF-7
-
-
- C H322
- F H322
- lv
- A1
- A1

5'-upstream regulatory sequences. It must therefore be regulated by either a post-transcriptional change or by the presence of regulatory sequences located within the processed transcriptional unit. This latter possibility is thought unlikely. Although enhancers are known to operate *in vitro* when positioned downstream, an enhancer located within the intronic sequences is more usual.

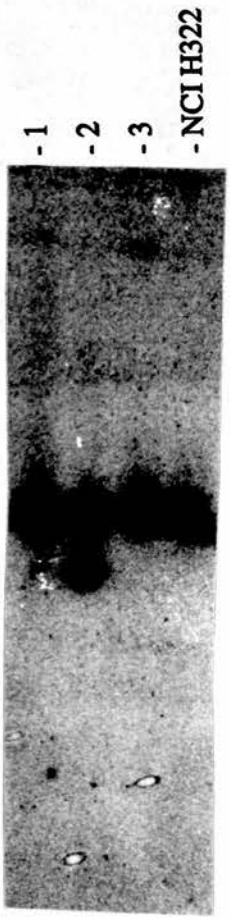
Concurring evidence comes from examination of the mRNA levels by Northern blot analysis, carried out on RNA prepared from cells grown in fresh or conditioned media. Figure 20 shows that levels of Pi class GST mRNA were not affected by the media used. Unfortunately the levels of Alpha class GST mRNA would require a more sensitive technique for mRNA detection. To gain a fuller understanding of the Alpha class GST induction it would also be important to measure the mRNA levels of the Alpha class GST induced in the MCF-7 transfectant. The induction of Pi class GST, must however result from a post transcriptional event, including increases in protein stability or translational efficiency, but excluding RNA stability. The simplest and most consistent hypothesis is that both Alpha and Pi class GSTs are co-ordinately regulated in a post-transcriptional manner. Further analysis will be required to fully understand the molecular basis of this response and the exogenous factors that mediate the induction.

V.6. Discussion

The NCI H322 cells express Pi and Alpha class GST at readily detectable levels. It is difficult to quantify the level of expression, relative to human tissues, since the levels vary with the culture conditions. It is however possible to compare which isoenzymes are present.

Figure 20: Levels of Pi class GST mRNA in conditioned NCI H322 cells.

Total RNA was prepared from three flasks of $5x10^6$ NCI H322 cells grown for 6 days. Media was changed; not at all (1); every other day (2); every day (3). (4) RNA from $2x10^7$ cells NCI H322 cells grown for 2 days at 37°C. 10 μg each RNA was electrophoresed alongside RNA. Fractionated RNA was transferred to Hybond-N before Northern blot analysis with a fragment of human GST-P1.


- 1
- 2
- 3
- NCI H322

The GSTs are approximately 20 fold less abundant in the lung than in the liver (Guthenberg and Mannervik, 1979). The predominant GST isozyme in the lung is GST-P1 (π). Alpha and Mu class GSTs are both found at lower concentrations (Carmichael *et al*, 1988; Cossar *et al*, 1990). Singal *et al* (1992) showed that the Alpha, Mu and Pi class isoenzymes represent 30%, 10% and 60% respectively, of the total GST protein isolated from lung. Although exact quantification of the levels is not possible, the relative proportions of the cytosolic GST classes in human lung tissue, correlate well with the relative GST proportions seen in NCI H322 cells.

The above studies however were all carried out on whole lung tissue. The lung is made up of a variety of cell types. The actual contribution of each of these cell types to detoxification in the lung is not known. There are also a wide variety of tumour types derived from different cell types in the lung. Coursin *et al* (1992) looked at the expression of GST isoenzymes in individual lung cell types using immunohistochemical analysis of tissue sections. They demonstrated that although Alpha and Mu class GST enzymes are found at low concentrations within the lung as a whole, some cells, notably Clara cells and alveolar type II cells stained quite strongly with an antiserum raised against the GST isoenzymes from human liver.

V.7.1. GST subunits in NCI H322 cells

NCI H322 cells appear to express several glutathione S-transferases. Alpha and Pi class GST subunits are both present in the cytosol in varying amounts (figure 11). By Western blot and immunohistochemical analysis Pi class GST appears to be the most abundant GST, although the Alpha class GST is also expressed at reasonable levels. Alpha class GST is found at

undetectable levels in low density frequently fed cultures whereas Pi class GST however appears to be present constitutively albeit at a lower level in the frequently fed cultures (figure 15). GST-M1 (μ) is not found expressed in these cells. Southern blot analysis revealed that these cells are not genotypically null for GST-M1 (figure 12). The polymorphism for which 50% of the population are homozygous for has been associated with an increased risk of lung cancer (Seidegard *et al*, 1986; Seidegard *et al*, 1990). The cells within the lung that express the GST-M1 (μ) in which this link was established were peripheral blood mononuclear leukocytes. Carmichael *et al* (1988) examined the presence of GST within lung samples and showed no GST-M1 in any of the samples. There was however a protein with a similar molecular weight as the protein I detect in the NCI H322 cells. Immunohistochemistry of human lung tissue with antibodies raised against GST-M1 show that Mu class GST is not found in all lung cell types. In lung, Alpha and Mu class Mu class GST staining has only been described in Clara cells and alveolar type II cells (Coursin *et al*, 1992). Other cells within the lung have not been found to express GST-M1. With the Mu class antisera M1-JN, M1-SP and M1-SL, a protein was detected in the NCI H322 cells on long exposures that ran with a similar mobility to the non polymorphic liver band, thought to be GST-M2. However it may not be the same protein, as two of the antisera that detect the faster liver protein, did not detect the similar mobility protein in the NCI H322 cells.

V.8.2. Identification of the cytosolic Mu-class GST

To further characterise the cytosolic Mu-class GST detected in the NCI H322 cells several methods could be used. Western blot analysis of two dimensional gels could be carried out. Another possibility is to establish

the HPLC profile of the protein. Both techniques are dependent on reasonable antibody detection of the protein. This is known to be difficult with the antisera in our laboratory. There were three antisera (M1-JN, M1-SP and M1-SL) which detected what appeared to be the same Mu class GST in the cytosols of NCI H322 cells. If the antisera were pooled, this might enhance the specific signal relative to the background. Some Mu class antisera have been shown to cross react poorly with other members of the Mu GST class. I have noted that only 4 out of 7 GST-M1 antisera detect the mouse Yb_1 GST used as a molecular weight standard. The converse is also true; not all antisera raised against rat Yb_1, detect the human GST-M1 (DeJong *et al* 1988b). Furthermore, Campbell *et al* (1990) noted that not all antisera raised against GST-M1 detected the brain and testis Mu class GST (GST5.2). Therefore, the Mu class antibodies may exhibit restricted cross antigen reactivity and the degree of cross reactivity detected for some Mu class GST subunits may be expected to be weak and to vary between preparations.

In whole lung, two Mu class GSTs have been described at low levels (Gupta *et al*, 1990; Singal *et al*, 1992). These two GSTs appear to be distinct from the Mu class GSTs of liver and muscle, as they display, lower CDNB activities, differing inhibitor kinetics and isoelectric points of 6.5 and 6.25 (Singal *et al*, 1992).

In future studies it may be possible to use specific antisera for other human Mu class GST subunits which might improve the detection of the NCI H322 protein. This, may establish the identity of the cytosolic protein as a Mu class GST, and enable HPLC and 2D-gel analysis to be carried out,

to allow further identification. A biochemical approach may be required to identify this protein, perhaps using a GST affinity column.

RNA PCR or Northern blot analysis might aid in establishing if this protein is a Mu-class GST. It would however be difficult to establish an association between a detected protein and its encoding mRNA. The mRNA levels might provide a diagnostic aid; however if the regulation of this protein is similar to GST-P1 and GST-A there is unlikely to be any change in the mRNA levels.

V.9.3. Identification of the Alpha class subunit(s)

It is not known which Alpha class subunits are present in the NCI H322 cells. There are only a few amino acid differences between GST subunits A1 and A2, so the antiserum does not distinguish between them. They both run with the same mobility. The heterodimer and homodimers of the two subunits can be differentiated by GSH sepharose affinity columns or reverse phase HPLC (Hayes *et al*, 1989). An alternative method would be to use specific oligonucleotide probes to detect the mRNA.

Analysis of the GST content of whole lung demonstrated the presence of three Alpha class isoenzymes (Singhal *et al*, 1992). These may represent the heterodimer and homodimers of the subunits A1 and A2.

The Alpha class subunits of the NCI H322 cells, demonstrated a slightly faster mobility than GST-A1 and GST-A2 from liver; in agreement with the Alpha class GSTs from whole lung (Carmichael *et al*, 1988). The cause of the difference is not known but suggestions include differences in

post translational modification. GSTs are known to be substrates for methylation, glycosylation and phosphorylation.

V.10.4. Regulation of the conditioning response

Glutathione S-transferase subunits from the Alpha, Mu and Pi classes, are all induced during normal cell culture. There are minor differences in the way Alpha and Pi class GSTs are regulated in the NCI H322 cells. Pi always appears expressed at detectable basal levels whereas Alpha and Mu class GSTs are not detectable in low density fed cultures. However the levels of all three classes were induced in cells growing at high density and at lower densities when subjected to media conditioned on high density cells. The likely inducing agent(s) was a diffusible factor of molecular weight less than 14 kD.

The conditioned media appeared to mediate a post-transcriptional response as shown by: 1) the Alpha class GST-A1 cDNA expressed from a heterologous promoter, responded to the conditioned media; 2) The levels of Pi class GST mRNA did not change, in conditions inducing the protein levels. Morrow *et al* (1992) have shown that a difference in the expression of GST-P1 between breast cancer cell lines with or without oestrogen receptor, is also post- transcriptional, however in that case it is effected by a stabilisation of the specific mRNA.

V.11.5. Heterogeneity of the conditioned response

The IHC had suggested the Alpha and Pi class GSTs are expressed at a high level only in a proportion of cells in confluent cultures. IHC with differently labelled antisera would be useful in establishing if the same cells

within the population are responsible for the induction of all three of the GST classes.

The clustering of cells with high levels of Alpha and Pi class GST (figure 5, p66) might be explained by two hypotheses: 1) the NCI H322 cells are not a homogeneous population and there is clonal variation in the induction of GSTs during normal growth; 2) there is some form of cell interaction between neighbouring cells. These two hypotheses may be differentiated between by cloning out from the NCI H322 cells. If the heterogeneity is not found within the clones, the clusters must be due to clonal variation. If however heterogeneity, in the same manner as in the original population of cells, is still existent within the clones; this would suggest cell-cell interactions.

The cells grown on conditioned media showed fairly low levels of induction (2-4 fold). It was impossible to used more highly conditioned media or to expose the cells for a longer period to the conditioned media, as the cultures deteriorated rapidly if left longer. When the cells were grown to high levels of confluence and fed frequently to keep them healthy, the levels of induction were at least 20 fold (figure 13). This may suggest the response is not entirely mediated through the culture media. It may partially be due directly to the density of the cultures, perhaps due to the numbers of cell interconnections. This might be tested by growing cells to high density, then scraping the majority of cells from the flask. This may enable differentiation between an effect due to total density of the cells in the flask and that due to the local density of the cells.

In future studies it would be interesting to establish the role of this response, the means by which it is mediated and its regulation.

CHAPTER VI

Analysis of GST expression during heat shock

VI. ANALYSIS OF GST EXPRESSION DURING HEAT SHOCK

The immunohistochemical analysis of the NCI H322 cells had suggested that the expression of the glutathione S-transferases changed both during and after heat shock (chapter III, p54). Both nuclear Mu and cytoplasmic Pi class GST staining appear to increase during a 2 day heat shock at 42°C. The levels then drop before returning to higher levels after a few days recovery. An increase in GSTs due to the conditioning media response described in chapter V may explain this second increase in Pi class GST expression. The Mu class GST seen with immunohistochemical analysis was nuclear. No nuclear Mu class GSTs were detected with the antisera used in Western blot analysis of the conditioning media response. As there was general difficulty in detecting the nuclear Mu class GST on western blots and as all three detectable GSTs in the cytosol are affected by the conditioning response it is quite plausible that the second increase in nuclear Mu class GST seen with immunohistochemistry was also due to the same response. The initial increase seen immediately after the heat shock may have been a direct response to heat shock.

VI.1. GST expression following 42°C heat shock

To investigate the effect of heat shock on GST expression, Western blot analysis of heat shocked NCI H322 cells was carried out. NCI H322 cells were seeded out in 175 cm^2 flasks at either $6x10^6$ or $2.5x10^7$ cells per flask. The cells were left 24 hours to recover before being placed at 42°C, 5% CO_2 in a humidified incubator for 48 hours. Following the treatment the cells were returned to 37°C to allow recovery. Flasks of cells were harvested throughout the experiment. During the experiment, the

morphology of the cells changed quite dramatically, the heat shocked cells became more flattened and developed many protrusions (figure 21, p132). This is thought to be due to dissociation of the actin microfilament stress fibres (Glass *et al*, 1985). There are changes within the structure of the nuclei, the morphology of the nucleoli has been reported to be altered during heat shock (Pelham, 1984). The cells plated at the lower density were more affected and took longer to begin dividing. It is difficult to estimate visually the amount of cell death if any caused by the heat shock but this treatment is considered to be mainly cytostatic rather than cytotoxic. After harvesting the cells were fractionated to make protein preparations from nuclei and cytoplasm.

Western blot analysis of the heat shocked samples, showed Alpha and Pi class GSTs to be present in varying levels in the cytosols of both control and heat shocked cells. This experiment was carried out before the investigation on the expression of GSTs during normal cell growth (described in chapter V). It was therefore impossible to correlate the levels with heat shock due to the effects of the conditioning media response.

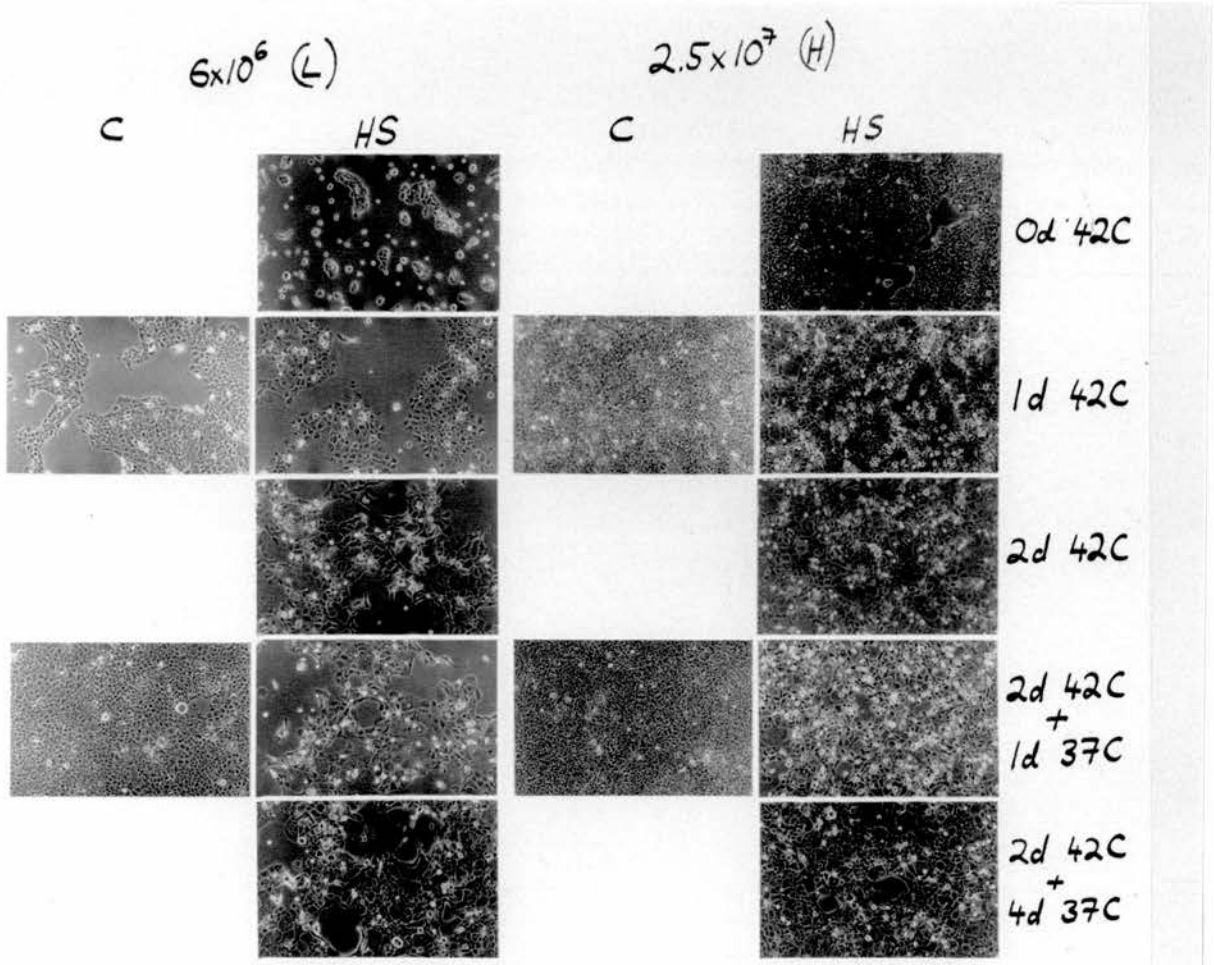
VI.1.1. Induction of a nuclear Mu-class GST during heat shock

The Mu-class antiserum M1-SH does not detect the presence of any Mu class GST in the cytosol. This is consistent with both the immunohistochemistry data (chapter III) and the expression data in chapter V where only in highly confluent, underfed cells was any Mu class GST detected (p106).

Immunohistochemical analysis had suggested the presence of a Mu class GST in the nucleus which was induced by heat shock. Western

Figure 21: Morphology of heat shocked NCI H322 cells

$6x10^6$ or $2.5x10^7$ NCI H322 cells were seeded and after 1 day at 37°C they were either exposed to 42°C for 48 hours and then allowed to recover at 37°C. Control cells were left at 37°C for the duration of the experiment. Cells were photographed under phase contrast microscopy (100x) before harvesting for Western blot analysis (figures 22 and 23).

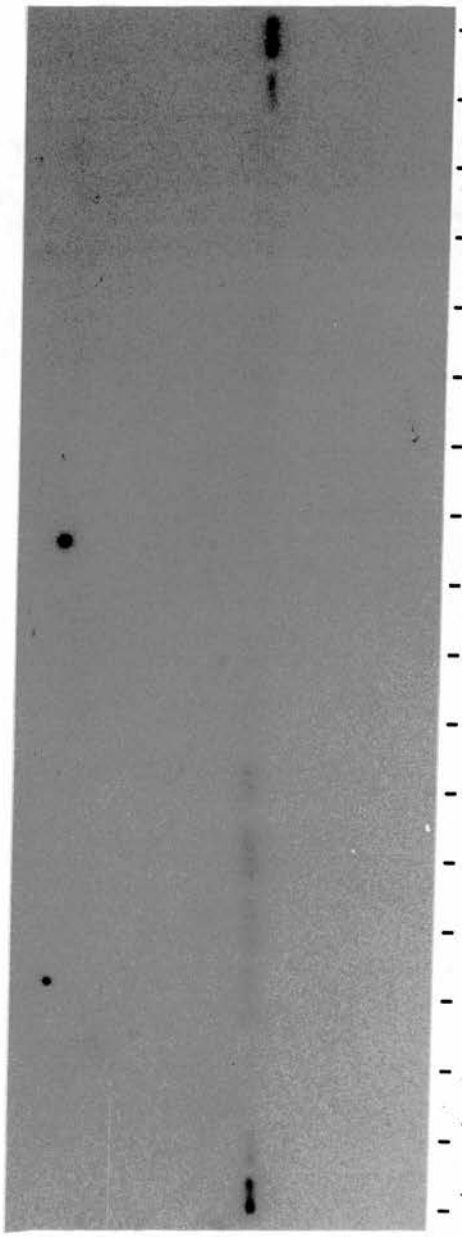

6x10^6 (L)
2.5x10^7 (H)
C
HS
C
HS
0d 42C
1d 42C
2d 42C
2d 42C + 1d 37C
2d 42C + 4d 37C

analysis using the Mu-class antiserum M1-SH (figure 22), detected a band with the same mobility as mouse Yb_1, present in the nucleus of the low density plated cells, both in the 1 and 2 day heat shocked cells, as well as in the 1 day recovery cells. It was also present at a lower level in the high density plated cells after 1 and 2 days at 42°C. One out of the four control samples also has this band present, the reason for this is not clear. The IHC was suggestive of a second induction peaking at day 5 and 6 but this was not detected with the Western blot analysis. The IHC detected Mu class staining in the nucleus of both normal and unstressed cells, the Western blot analysis however only detected staining after heat shock although it was detectable in some of the control cells. It is probable that as the protein was poorly detectable with Western blot analysis it may be present in all the Western blot control samples at a subdetectable level with one out of the four samples having higher levels. One possibility is that this putative nuclear Mu class GST is induced by the same conditioned media response seen with the cytosolic GSTs. This may explain the induction seen with IHC, 5 days after cells were heat shocked, although the IHC suggested the levels then dropped. Improved detection would enable this to be clarified.

Several different antisera, raised against human and rat Mu-class GSTs, were used. The antiserum M1-SH was the only one that detected the band convincingly although this was only on long exposures; but even this was not consistent. The conditions of the western blot analysis were altered to try and improve the sensitivity. This resulted in decreased specificity of binding, with the desired band becoming undetectable from the background. Mu class GST staining was not detectable in the cytoplasm. A sensitive method of detecting mRNA transcripts is the use of coupled reverse transcription and PCR methodology. Oligonucleotides made to the most

Figure 22: Western blot analysis of Mu class GST staining in the nucleus of cells heat shocked at 42°C.

$6x10^6$ (1) or $2.5x10^7$ (2) NCI H322 cells were seeded and after 1 day at 37°C they were exposed to 42°C for 24 (1d 42°C) or 48 hours (2d 42°C). Cells exposed to 48 hours 42°C were allowed to recover at 37°C for 1, 4, 6 or 8 days recovery (1d, 4d, 6d, 8d R). Control cells were grown for 2 (C2) or 4 (C4) days at 37°C. Cells were harvested and nuclear and cytosol protein fractions were prepared. 40μg nuclear protein samples were subjected to SDS-PAGE, transferred to nitrocellulose and Western blotted with Mu class GST antiserum (SH). Mouse Mu class GST (Yb_1) was run alongside the samples.


- Yb1 (100ng)
- Yb1 (20ng)
- 4d R [2]
- 1d R [2]
- 2d 42C [2]
- 1d 42C [2]
- 37C (C4) [2]
- 37C (C2) [2]
- 8d R [1]
- 6d R [1]
- 4d R [1]
- 1d R [1]
- 2d 42C [1]
- 1d 42C [1]
- 37C (C4) [1]
- 37C (C2) [1]
- Yb1 (20ng)
- Yb1 (100ng)

highly conserved regions of the Mu-class genes were used to detect any Mu class GST mRNAs expressed in these cells. GST Mu class mRNAs were detected in rat and human liver but not in RNA prepared from NCI H322 cells either grown at 37°C or 42°C for 48hr. This experiment can not be shown to be indicative of lack of Mu class mRNA expression. The 22 nucleotide oligonucleotides used had 3 or 4 mismatches with some known Mu class GST sequences. It might be possible to alter the conditions to enable better detection of less homologous sequences.

VI.1.2. Pi class GST cross reactivity in the nucleus of heat shocked cells

As expected from the immunohistochemistry, the Pi class GST antiserum (P1-λ) detected no 24 kD band in the nucleus (figure 23). However it did detect several bands of approximately 40-50 kD. Changes were seen in these high molecular weight bands in the nucleus that appeared to alter with the heat shock, especially those in the 40-45kD range. Higher levels were detected shortly after the 2 day 42°C heat shock and had returned to control levels by eight days following the heat shock.

The size of the detected protein suggested an unresolved GST dimer. An attempt to show the specificity of the antibody binding to the high molecular weight nuclear protein was tested by antibody depletion. Varying amounts of purified Pi class GST antigen were mixed with the antiserum to deplete its binding capacity. Figure 24 shows that although partial depletion of the P1-λ antiserum by the mouse Yf protein was observed there was no observed depletion for the nuclear protein. If depletion of the antiserum had resulted in loss of binding for the high molecular weight protein this would have shown that the binding was specific. However, inability to observe

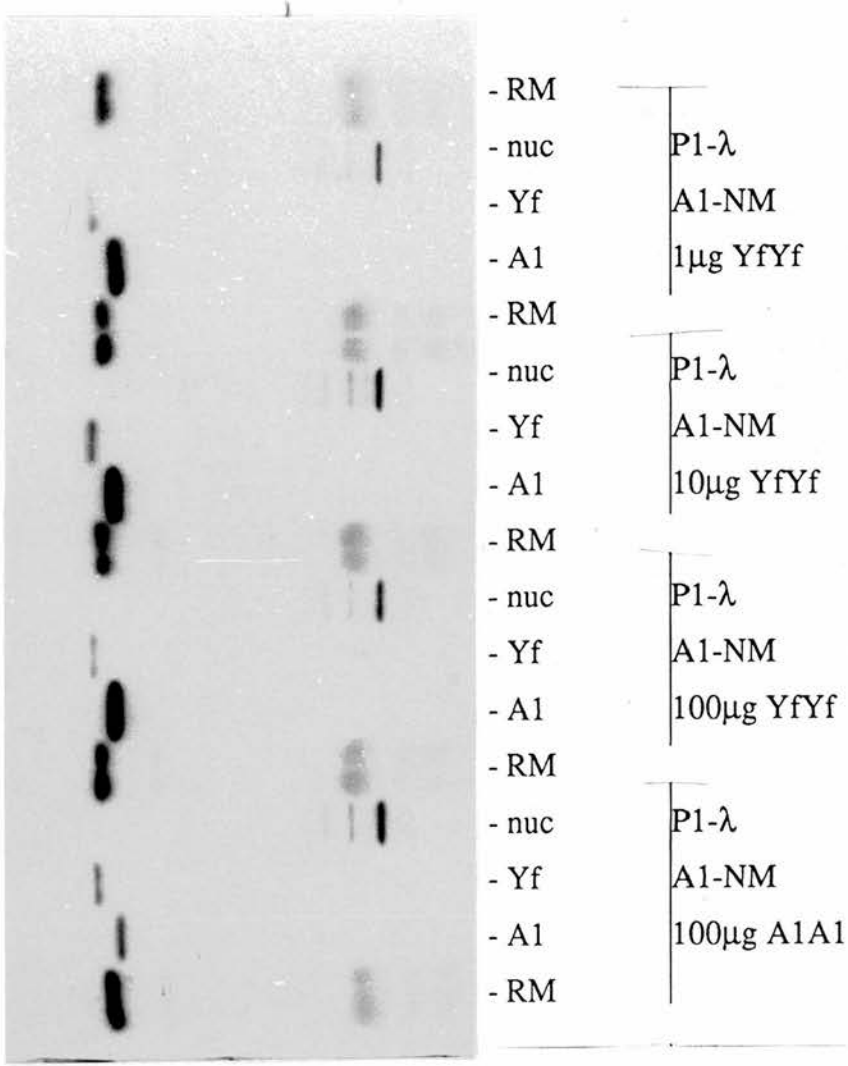
Figure 23: Western blot analysis of Pi class GST in cells heat shocked at 42ºC.

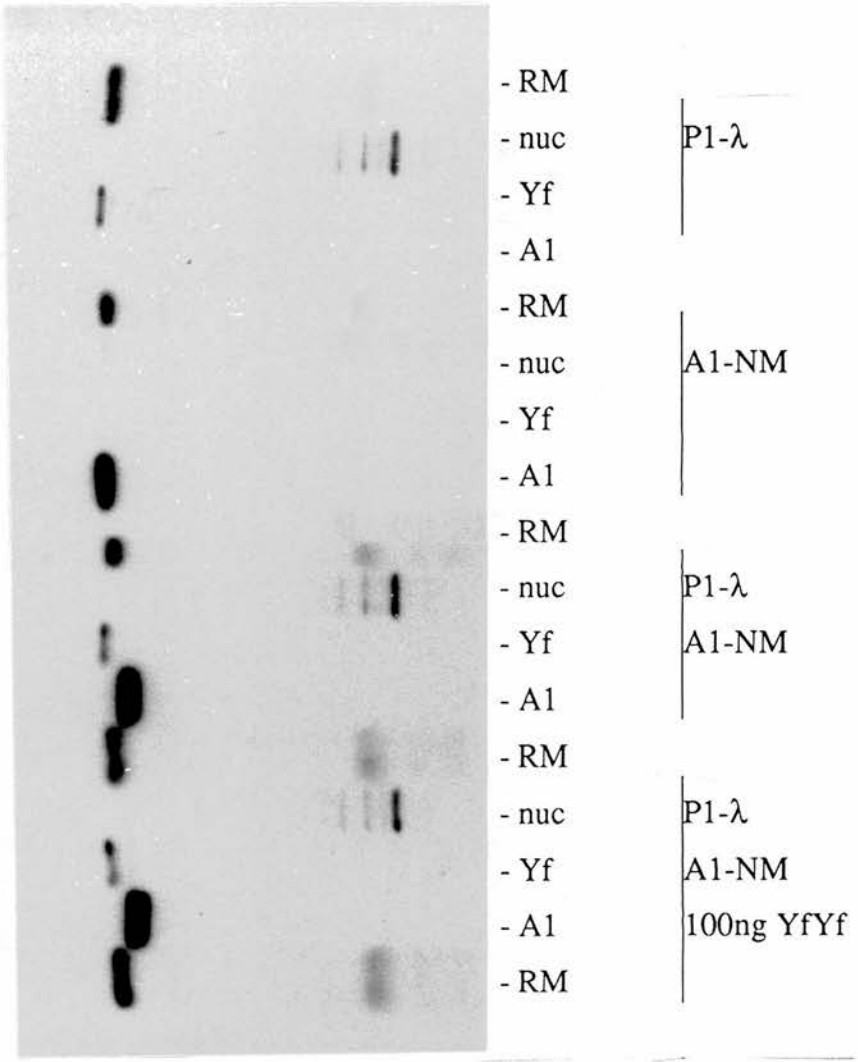
$6x10^6$ (1) or $2.5x10^7$ (2) NCI H322 cells were seeded and after 1 day at 37°C they were exposed to 42°C for 24 (1d 42°C) or 48 hours (2d 42°C). Cells exposed to 48 hours 42°C were allowed to recover at 37°C for 1, 4, 6 or 8 days recovery (1,4,6,8d R). Control cells were grown for 2 (C2) or 4 (C4) days at 37°C. Cells were harvested and nuclear protein fractions were prepared. 40µg nuclear protein samples were subjected to SDS-PAGE, transferred to nitrocellulose and Western blotted with Pi class GST antiserum. Human liver (lv) and the GST standard mouse Pi class GST (Yf) were run alongside the samples.

- lv
- Yf (20ng)
- (C2) 37C [1]
- (C4) 37C [1]
- 1d 42C [1]
- 2d 42C [1]
- 1d R [1]
- 4d R [1]
- 6d R [1]
P1-λ
- 8d R [1]
- (C2) 37C [2]
- (C4) 37C [2]
- 1d 42C [2]
- 2d 42C [2]
- 1d R [2]
- 4d R [2]
- (20ng) Yf
- (100ng) Yf
- (500ng) Yf

Figure 24; Depletion of Pi class GST antisera.

20 μg nuclear protein from NCI H322 cells was fractionated by SDS-PAGE alongside 20 ng of mouse YfYf and human A1-A1 GST isoenzymes. Eight panels were run separated by molecular weight markers. Western blot analysis was then carried out with each panel incubated with the following preincubated antisera mixtures. Alpha and/or Pi class GST antisera were preincubated with one of the following; 0, 100 ng, 1 μg, 10μg or 100μg mouse Yf or 100μg human GST-A1. Preincubations were carried out for 60 min, 20°C with gentle shaking.


- RM
- nuc
- Yf
- A1
P1-λ
A1-NM
1µg YfYf
- RM
- nuc
- Yf
- A1
P1-λ
A1-NM
10µg YfYf
- RM
- nuc
- Yf
- A1
P1-λ
A1-NM
100µg YfYf
- RM
- nuc
- Yf
- A1
- RM
P1-λ
A1-NM
100µg A1A1

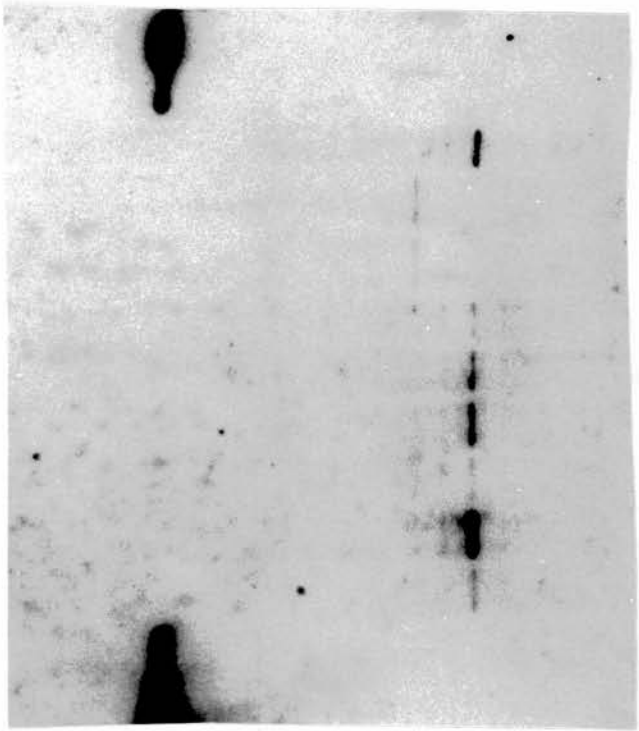

- RM
- nuc
- Yf
- A1
P1-λ
- RM
- nuc
- Yf
- A1
A1-NM
- RM
- nuc
- Yf
- A1
P1-λ
A1-NM
- RM
- nuc
- Yf
- A1
- RM
P1-λ
A1-NM
100ng YfYf

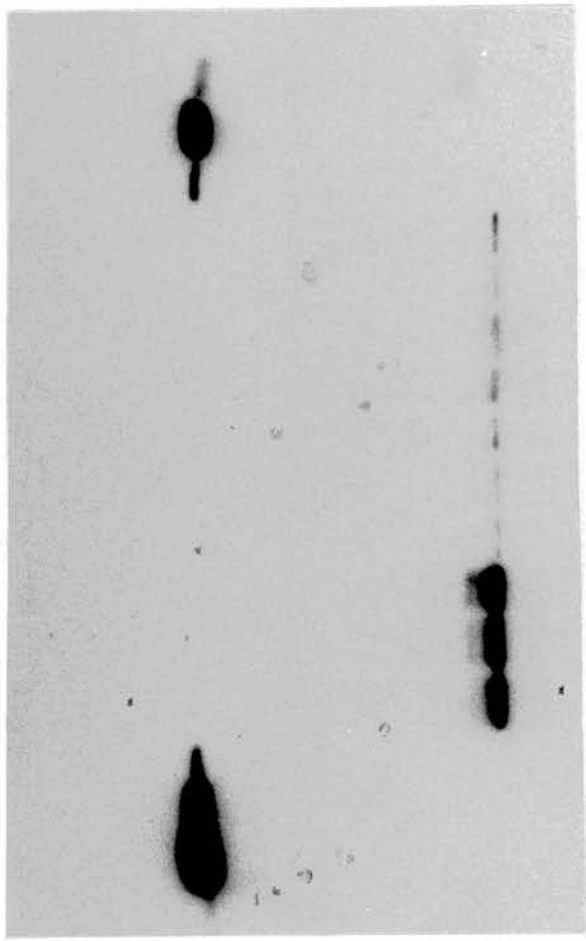
loss of binding would not prove the reverse, since the protein used to deplete the human GST-P1 antibodies from the antiserum was the mouse homologue, Yf. The human and mouse Pi class GSTs are highly homologous, but can be expected to show some differences at their antigenic sites. So although a polyclonal antiserum cross reacts very well between the species, there may some antibodies that are species specific and would therefore not be depleted from the serum. If there are species specific GST-P1 antibodies present within the antiserum that are detecting the high molecular weight protein it is possible that the detected protein is related to GST-P1. Depletion of the antiserum with the antigen that the antisera was raised against would establish this.

To test if the high molecular weight band was a complex of protein resistant to SDS denaturation an attempt was made to disrupt it with a variety of treatments (figure 25). None of the standard treatments for disrupting a non covalent bond altered the molecular weight of the nuclear band. However when the protein samples were incubated with 10 mM glutathione, the molecular weight of the band was altered, abundance of the highest molecular weight band decreased and the lowest molecular weight band increased (figure 25). However, there was no appearance of a 24 kD subunit. The change in molecular weight only represents a difference of about 8 kD, not enough to suggest a GST subunit. If glutathione treatment affected the protein by interacting via a glutathione binding site, glutathione analogues should be equally affecting, however, addition of S-hexyl glutathione did not alter the molecular weight of the protein. The nature of this protein was not investigated further.

Figure 25: Use of disruption methods on GST Pi immunoreactive protein.

Nuclear protein samples from control NCI H322 cells (C) and cells harvested after 6 days recovery from a 2 day 42°C heat shock (HS) (see figure 23) were treated with various disrupting methods, 1) (C) standard boiling mix. 2) (C) standard boiling mix + 20 min 100°C. 3) (C) standard boiling mix + 5 hr 55°C. 4) (C) standard boiling mix + 10mM DTT, 40 min 20°C. 5) (C) standard boiling mix + 10mM DTT, 40 min 20°C + 5 min 100°C. 6) (C) standard boiling mix + 1% TFA, 40 min 20°C + 6 µl 1M Tris-base. 7) (C) standard boiling mix + 1% TFA, 40 min 20°C + 6 µl 1M Tris-base + 5 min 100°C. 8) (HS) standard boiling mix. 9) (HS) standard boiling mix + 1% TFA, 40 min 20°C + 6 µl 1M Tris-base. 10) (HS) standard boiling mix + 1% TFA, 40 min 20°C + 6 µl 1M Tris-base, 5 min 100°C. 11) (C) standard boiling mix. 12) (C) 10 mM GSH, 80 min 37°C + standard boiling mix. 13) (C) 10 mM GSH, 80 min 37°C + standard boiling mix, 5 min 100°C. 14) (C) 10% EtOH, 80 min 37°C + standard boiling mix. 15) (C) 10% EtOH, 80 min 37°C + standard boiling mix, 5 min 100°C. 16) (C) 1 mM S-hexyl-GSH, 80 min 37°C + standard boiling mix. 17) (C) 1 mM S-hexyl-GSH, 80 min 37°C + standard boiling mix, 5 min 100°C. 18) (C) 10 mM S-hexyl-GSH, 80 min 37°C + standard boiling mix. 19) (C) 10 mM S-hexyl-GSH, 80 min 37°C + standard boiling mix, 5 min 100°C. Samples were then fractionated on SDS-PAGE on a 12% gel. GST standard mouse Yf was run alongside the samples. Western blot analysis was carried out with antisera P1-λ raised against human GST-P1.


- Yf (100ng)
- Yf (20ng)
- 11
- 12
- 13
- 14
- 15
- 16
- 17
- 18
- 19
- Yf (20ng)
- Yf (100ng)


- lv
- Yf (100ng)
- Yf (20ng)
- 1
- 2
- 3
- 4
- 5
- 6
- 7
- 8
- 9
- 10
- Yf (20ng)
- Yf (100ng)
- Yf (500ng)

VI.1.3. GST expression following 42ºC heat shock, allowing for induction of GST by the conditioning response

After the expression of GST in normal cells had been studied, the heat shock at 42°C was repeated (figure 26). This time the cells were fed daily and cells harvested for control samples every day. During the two day heat shock the Alpha class GST did not change. The levels then appeared to drop to an undetectable level, while the control GST levels increased as expected, as the cells become highly confluent. The levels in the heat shocked cells did not appear to increase as the cells started to grow again. Pi GST levels increase during the 2 days at 42°C by about 2 fold. The Pi class GST levels then return to the initial levels within 2 days. They do not drop below the initial levels as in the case of the Alpha class GST.

VI.1.4. Lack of transcription of Pi class GST in heat shocked cells

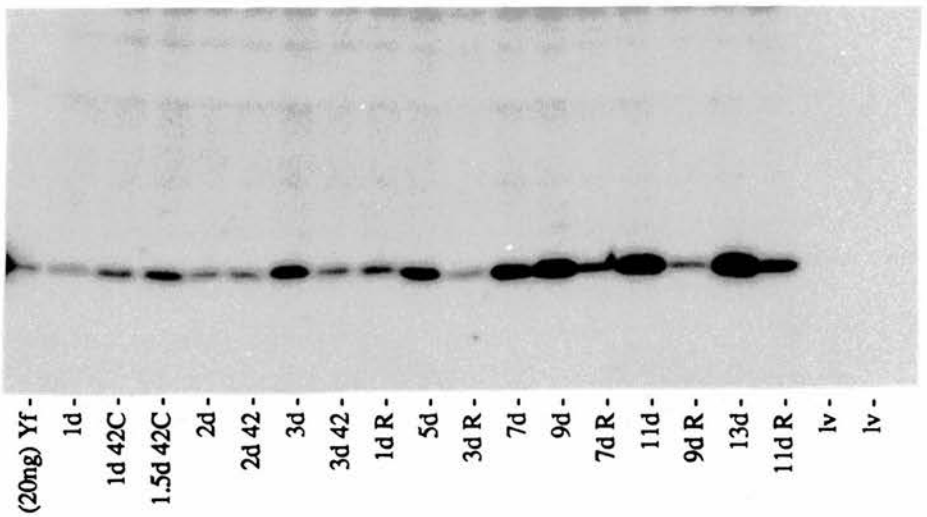
RNA, from cells stressed for 48 hours at 42°C, was analysed by Northern blot (figure 27). The levels of Pi class GST mRNA, drop significantly in the heat shocked cells. Mu class GST RNA was undetectable and Alpha class GST RNA levels were barely detectable only in the control sample.

VI.2. 45ºC heat shock of NCI H322 cells

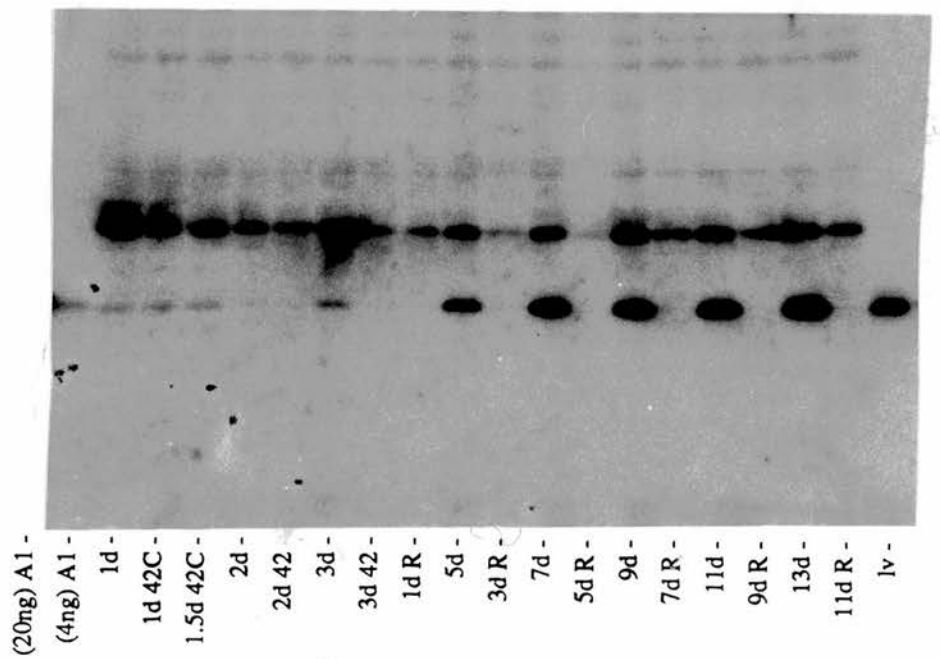
As the 42°C heat shock involved long exposure times it was decided to carry out a higher temperature heat-shock at 45°C. This would involve shorter exposure times and minimal effects on the GST levels due to the conditioning media response. Cells were seeded in 175cm^2 flasks and

Figure 26: Expression of GST in cytosol of 42ºC heat shocked NCI H322 cells.

NCI H322 cells were placed at 42°C for 1, 2 or 3 days (d 42°C). Cells were allowed to recover for 1 to 11 days following 2 days at 42°C (d R). Control cells were harvested on the same days as samples (d). 20μg cytosolic protein was fractionated by SDS-PAGE and transferred to nitrocellulose before Western blot analysis with human GST-A1 and P1 antisera.


(20ng) Yf -
1d -
1d 42C -
1.5d 42C -
2d -
2d 42 -
3d -
3d 42 -
1d R -
5d -
3d R -
7d -
9d -
7d R -
11d -
9d R -
13d -
11d R -
lv -
lv -

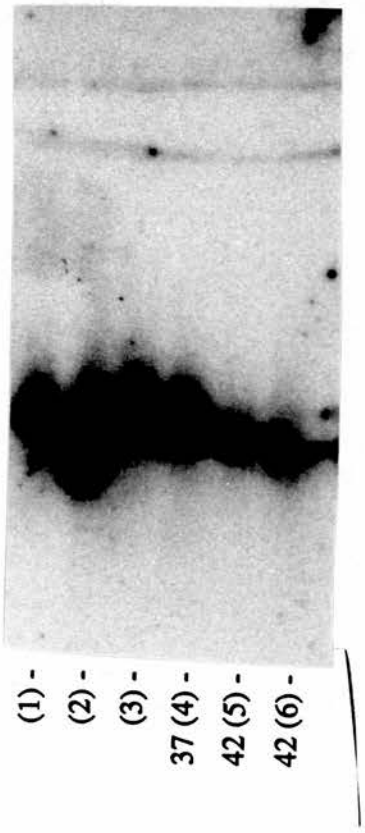
GST-P1


(20ng) A1 -
(4ng) A1 -
1d -
1d 42C -
1.5d 42C -
2d -
2d 42
3d -
3d 42 -
1d R -
5d -
3d R -
7d -
5d R -
9d -
7d R -
11d -
9d R -
13d -
11d R -
lv -

GST-A1

Figure 27: Northern analysis of 42ºC heat shocked cells.

1 x 10^7 (4, 5) or 2 x 10^7 (6) NCI H322 cells were seeded in 175 cm^2 flasks and allowed to grow at 37°C for 2 days. Flasks 5 and 6 were placed at 42°C for 48 hours, following which RNA was prepared from all three flasks. 10µg each RNA was fractionated alongside 10µg RNA's from control NCI H322 cells grown at 37°C for 6 days (1,2,3). Fractionated RNA was transferred to Hybond-N before Northern-blot analysis with a human GST-P1 DNA fragment.


(1) -
(2) -
(3) -
37 (4) -
42 (5) -
42 (6) -

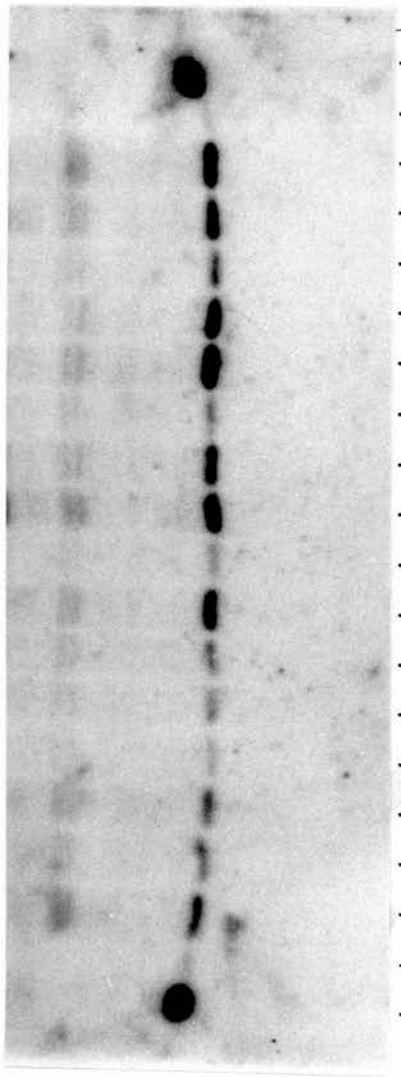
allowed to settle at 37°C for two days before being placed at 45°C for either 60, 90 or 120 minutes. The cells were then allowed to recover for either 0, 2, 4, 6 or 8 hours before harvesting. There was no increase in Pi class GST with the 60 minute shock (figure 28). After 90 minutes at 45°C Pi class GST is induced 2 fold over the 37°C control. By 8 hours recovery Pi class GST levels had returned to normal. The amount of protein loaded in the 2 hour recovery sample is low so it is difficult to estimate whether this represented a real drop in Pi class GST levels. After the 120 minutes at 45°C, the Pi class GST levels increase about 4 fold by 8 hours following the heat shock.

VI.3. Effect of heat shock on the drug sensitivity of NCI H322 cells

A cytotoxicity assay was carried out with heat shocked and control cells to establish if heat shock affected the sensitivity of the cells to cytotoxic drugs. Cells were seeded at a density of $2x10^4$ per well in 96 well plates. They were then subjected to 0, 30, 45 or 60 minutes at 45°C and left 3 hours before being challenged with several concentrations of adriamycin, CDNB, ethacrynic acid or menadione. The results (figure 29) show that no change in cell sensitivity to these drugs occurred as a result of the heat shock. It is not known how long the cells might take to develop any change in resistance to cytotoxic drugs as a result of the heat shock. As this experiment only used a single recovery time of 3 hours, another cytotoxicity assay was carried out using a heat shock of 60 minutes, followed by varying recovery periods, before exposing the cells to either adriamycin or CDNB (figure 30). Immediately after the heat shock, the cells showed a decrease in sensitivity to adriamycin, which lasted for between 1 and 2 hours after heat

Figure 28: Expression of GST in 45ºC heat shocked NCI H322 cells.

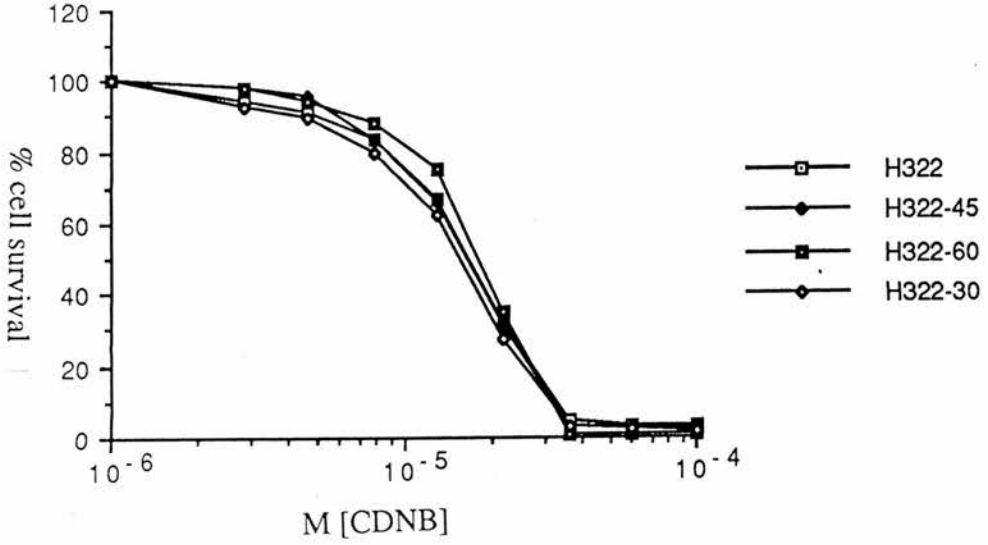
NCI H322 cells were seeded in 175 cm^2 flasks at a density of 2 x 10^7 cells. Two days later cells were placed at 45°C for 0, 60, 90 or 120 minutes. The cells were then returned to 37°C for 0, 2, 4, 6 or 8 hours. Cells were harvested and cytosolic protein was prepared. Methods are as described in chapter II. 20 μg cytosolic samples were subjected to SDS-PAGE, transferred to nitrocellulose and Western blotted with an antiserum raised against Pi (P1-λ) class GST. Rainbow markers and the GST standard, mouse Pi class GST (Yf) was run alongside the samples.

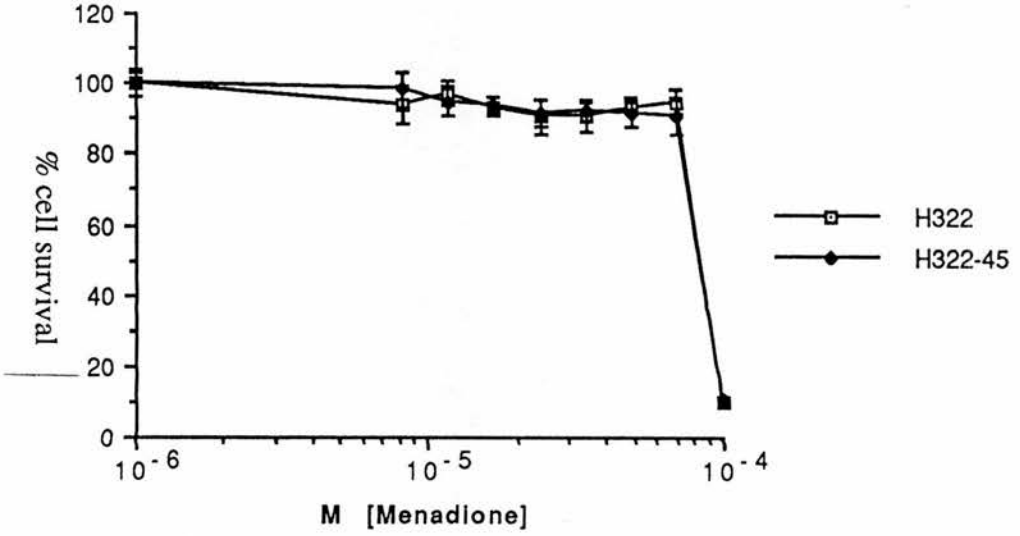


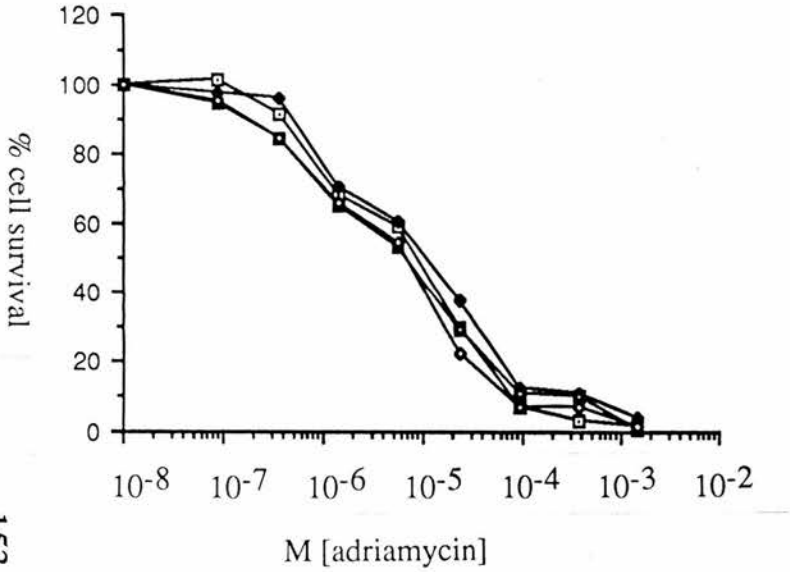
- RM
- Yf (20ng)
- 120min 45C - 8h 37C
- 120min 45C - 6h 37C
- 120min 45C - 4h 37C
- 120min 45C - 2h 37C
- 120min 45C - 0h 37C
- 90min 45C - 8h 37C
- 90min 45C - 6h 37C
- 90min 45C - 4h 37C
- 90min 45C - 2h 37C P1-λ
- 90min 45C - 0h 37C
- 60min 45C - 8h 37C
- 60min 45C - 6h 37C
- 60min 45C - 4h 37C
- 60min 45C - 2h 37C
- 60min 45C - 0h 37C
- 0min 45C - 8h 37C
- Yf (20ng)
- RM

Figure 29: Effect of heat shock on NCI H322 cells' drug sensitivity.

2×10^4 cells per well were seeded in 96 well plates. These were exposed to 45°C for 30, 45 or 60 minutes or left at 37°C. Cells were then returned to 37°C and after 3 hours, were exposed to varying drug concentrations for 2 hours. The cells were then washed and fed with fresh media. After 5 days, the numbers of viable cells were measured using the MTT assay as described in chapter II, p38. Percentage cell survival was plotted against drug concentration.


120
100
80
60
40
20
0
% cell survival
10-6
10-5
10-4
M [CDNB]
H322
H322-45
H322-60
H322-30


120
100
80
60
40
20
0
% cell survival
10-6
10-5
10-4
M [Menadione]
H322
H322-45


120
100
80
60
40
20
0
% cell survival
10-8
10-7
10-6
10-5
10-4
10-3
10-2
M [adriamycin]

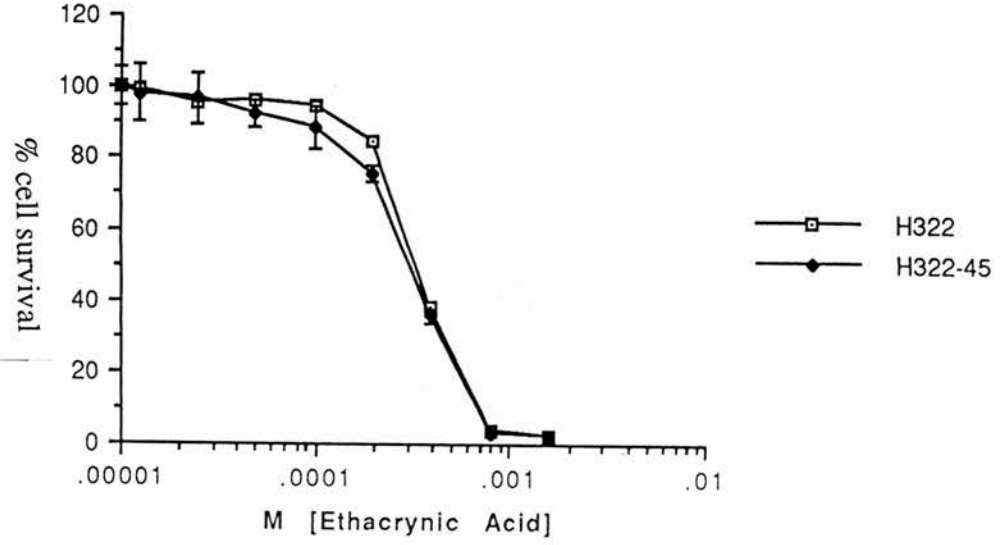
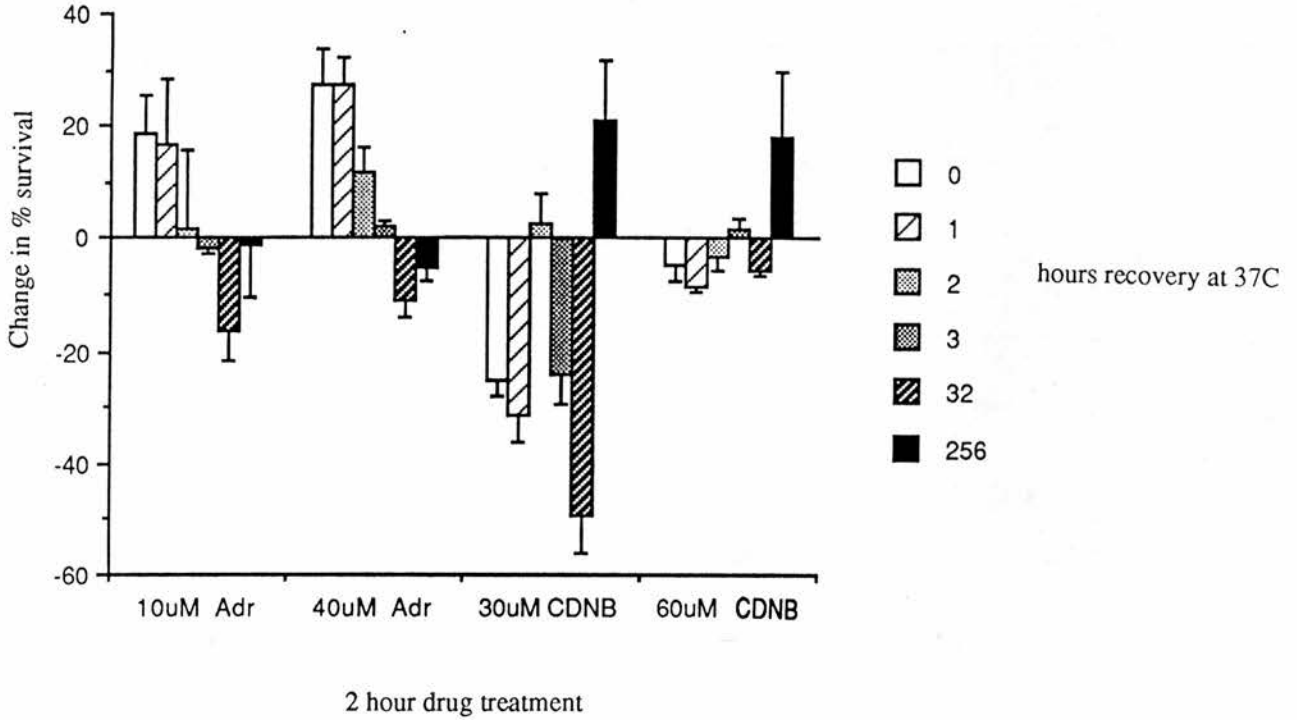

120
100
80
60
40
20
0
% cell survival
.00001
.0001
.001
.01
M [Ethacrynic Acid]
H322
H322-45

Figure 30: Effect of heat shock and varying recovery period on the drug sensitivity of NCI H322 cells.

2×10^4 cells per well were seeded in 96 well plates. These were exposed to 45°C for 60 minutes or left at 37°C. Cells were then returned to 37°C, and after a varying period at 37°C, were exposed to 10 or 40 μM Adriamycin, or 30 or 60 μM CDNB for 2 hours. The cells were then washed and fed with fresh media. After 5 days, the numbers of viable cells were measured using the MTT assay as described in chapter II, p38. The change in percentage cell survival between heat shocked and no heat shocked cells, was plotted with the recovery period.


40
20
0
-20
-40
-60
Change in % survival
10uM Adr
40uM Adr
30uM CDNB
60uM CDNB
2 hour drug treatment
0
1
2
3
32
256
hours recovery at 37C

shock, with a subsequent return to control levels. This could be due to an increased drug resistance mechanism or, if the heat shock inhibited the growth of the cells the adriamycin may have been less effective on slower growing cells resulting in an apparent increase in resistance. The normal sensitivity returned after 2 hours and by 32 hours after heat shock the cells were then found to have an increased sensitivity of approximately 15% compared with the non heat shocked cells. When CDNB was added to the heat shocked cells they appeared to become more sensitive.

VI.4. Discussion

VI.5.1. Pi class GST as a heat shock protein

Pi class GST has been shown to be induced 2-4 fold in heat shocked NCI H322 cells both with a long 2 day exposure at 42°C or a shorter 90 minute exposure at 45°C. Pi class mRNA levels are not induced and are found at very low levels in the cells kept at 42°C for 2 days. Chapter V showed that Alpha and Pi class GSTs can be regulated post-transcriptionally. The increase in Pi class GST protein may be a result of similar post transcriptional events. One reason may be increased protein stability relative to other proteins within the cells. One of the proteins induced by heat shock is ubiquitin which covalently attaches to proteins. This ubiquitination targets proteins for degradation (reviewed by Ang *et al*, 1992). This may act as a selection for increasing the stability of some proteins relative to others within the cell.

High molecular weight proteins (40-50 kD) were detected in the nuclear fraction of the NCI H322 cells using a GST-P1 antisera. The levels

of these were also altered by heat shock. However the relationship between these nuclear proteins and GST-P1 was difficult to establish. The immunological data did not suggest a relationship, however incubation of the high molecular weight nuclear protein with glutathione appeared to alter the molecular weight of the protein. The nuclear structure and protein content is greatly affected by heat shock (Pelham, 1984; Warters *et al*, 1986)

The timing of the Pi class GST response to heat shock varied between the IHC and the Western blot analysis. With the IHC the Pi class GST was seen to increase after 5 days recovery (figure 3). With the Western blot analysis Pi class GST was not seen to increase again until 11 days after the heat shock (figure 26). This is probably due to differences in the level of stress suffered by the cells in the different experiments and how long they took to recover and grow to sufficient confluence levels to express increased levels of GST-Pi. The cells grow quite differently in the different flasks possibly as a result of different gas exchange through the cap. Also there were variations in the numbers of cells per cm^2 and the cells per ml of media, both of which would affect the degree of stress. The size of the flask and the volume of media within will also affect the speed at which the cells heat up and cool down. The heat shock was carried out for 48 hours so the proportion of this time in which the cells were warming up and cooling is relatively small, this would not be expected to affect the length of time the cells were at 42°C. However the rate at which cells are heated does have an effect on the response. Cells which have been heated up slowly have higher levels of non-hsp protein synthesis and lower levels of hsp synthesis (Tomasovic and Koval, 1985; Burns *et al*, 1986). These cells also display higher levels of thermotolerance.

VI.6.2. A novel nuclear Mu class GST

A novel heat inducible putative Mu class GST isoenzyme has been detected in the nucleus. This protein was only detected with Western blot analysis after the cells had been heat shocked, although one of the control samples also had low levels (figure 22, p135). No nuclear Mu class GSTs were detected with the antisera used when studying changes in the expression of GST due to the conditioning media response. During IHC analysis the Mu class GST staining in the nucleus was detectable in all samples, although at varying levels. This discrepancy may have simply been due to poor detection of the protein by the available antisera used in Western blot analysis, possibly a result of the different conformational state of the protein.

It is likely that the detected protein was a Mu-class GST since it was detected by two antisera raised against different protein preparations and against different allelic forms of the hepatic isoenzyme (GST-M1a and GST-M1b). However five other polyclonal antisera raised against GST-M1 do not detect the protein. There also seems to be a difference between detection on Western blots and detection with immunohistochemistry. The antiserum used for the immunohistochemistry, M1-MR is not seen to detect the nuclear protein with Western blot analysis. Also the antiserum used for the Western blot detection (M1-SH) did not detect the protein every time.

There are several reasons as to why the nuclear protein was not detected with western blot analysis on every occasion; 1) poor Ab cross reactivity; 2) differences in the conformational state of the antigen; and 3) loss of protein from the nuclear fraction.

VI.7.3. Are the GST subunits detected in the nucleus contaminants from the cytoplasm?

The presence of a nuclear GST has been reported. Bennett *et al* (1982, 1986) and Tan *et al* (1988) demonstrated the presence of GST subunits within the nucleus, which has generated much discussion (Hayes *et al*, 1990). Several of the GST subunits found by Bennett *et al* (1986) and Tan *et al* (1988) were subunits also found at reasonable levels in the cytoplasm. It was suggested that the presence of GST within the nucleus maybe a result of contamination from the cytoplasm during cell fractionation (T. Mantle, GST and Drug Resistance Conference, Edinburgh, 1990).

During cell fractionation there is a tendency for proteins to diffuse between the nucleus and the cytoplasm especially when homogenised in aqueous buffers of pH 7-8. This however does not seem to be the case here as the nuclear protein detected in the NCI H322 cells with the Mu class antibodies was not detected in the cytoplasm by Western blot analysis. With the IHC the staining was very clearly limited only to the nucleus of the cell (figure 2). For the protein detected here such an explanation would require selective sequestration of the protein during fractionation.

Non histone protein BA is found with IHC to be located in the nucleus to the interchromatinic regions as well as in the soluble nuclear and cytoplasmic regions of the cells. This protein has been shown to be a rat Yb GST (Bennett *et al*, 1986). When Bennett and Yeoman (1987) injected the Yb subunits isolated from nuclei they were found to reassociate with the nucleus. About 40% of the subunits found in the nucleus where associated

with the non soluble residue fraction suggesting association with the chromatin.

Tan *et al* (1988) have shown the nuclear presence of a rat GST subunit, termed GST 5*-5*, similar to the Theta class rat GST 5-5. Although the relationship of GST-5* to GST-5 is not clear, GST-5* has not yet been described in the cytoplasm. GST 5*-5* has been shown to have activity against DNA hydroperoxides and has a concentration of 140 000 molecules / nuclei. This seems too high to be merely contamination from the cytoplasm. Other data which argues for a nuclear location of GST subunits are as follows: 1) Tirminstein and Reed (1988; 1989) showed a GSH dependant peroxidase activity associated with the nucleus which they partially purified and showed to be a glutathione S-transferase. 2) A rat nuclear protein was identified on the basis of peptide mapping and pI as a Mu class heterodimer of Yb and Yb' (Bennett *et al,* 1986).

Coursin *et al* (1992) showed staining with an antisera raised against the liver GST isoenzymes in the nuclei of Clara cells in normal lung tissue sections. The preparation used to prepare the antisera contains both Alpha and Mu class GSTs. The NCI H322 cells are thought to originate from Clara cells (Falzon *et al*, 1986). The nuclear staining in the Clara cells may represent the same protein as I detected in the nucleus with IHC and Western blot analysis.

The Western blot analysis suggested the 26kD nuclear GST staining protein was only present in the nuclear fraction of the NCI H322 cells (figure 22). No staining was observed in the cytoplasm. The confocal microscopy of the IHC staining for Mu class GST demonstrated quite clearly the presence of staining within the nucleus and the absence of staining in the

cytoplasm (figure 2). These data all are suggestive of the presence of GST in the nucleus.

VI.8.4. The role of GST in the nucleus

There is a potential role for GST in the nucleus. The nucleus is one of the parts of the cell most sensitive to the effects of cytotoxic agents. It would therefore make sense for the cell to have a detoxification mechanism within the nucleus as a backup against any reactive compounds which have escaped the other detoxification mechanisms, especially those such as short lived radicals which tend to cause most damage close to where they are formed.

Bellomo *et al* (1992) demonstrate the presence of a nuclear / cytoplasmic GSH gradient by fluorescent labelling, with some evidence for active transport. GSH is already known to be sequestered in the mitochondrial matrix where it is actively transported. There may be a similar system working in the nucleus. GSH within the nucleus is not however indicative of a role for GST within the nucleus, GSH is involved with many other functions. However GSTs have been shown to have activity for DNA hydroperoxides (Tan *et al*, 1988).

GST has been shown to inhibit the formation of benzo(a)pyrene-DNA adducts both *in vitro* and *in vivo* (Hesse *et al*, 1982; Jernstrom *et al*, 1982). GST may act as a repair enzyme within the nucleus. The rat Yb_3 subunit has been shown to associate with DNA *in vitro* (Catino *et al* 1978; Bennett *et al*, 1982).

Tirmistein and Reed (1989) showed a lipid hydroperoxide activity associated with a partially purified GST from rat liver nuclei. GSH has been shown to inhibit lipid peroxidation in isolated rat nuclei (Belouqui and Calderbaum, 1986).

To characterise the Mu class GST induced by heat shock in the nucleus, a similar approach using 2D gel electrophoresis or HPLC profile as was suggested for the cytosolic protein could be used (chapter V, p123). The major problem is the lack of a good antibody. Better detection might be possible by establishing what causes the inconsistent detection (see p158). Problems with the native state of the protein or with cell fractionation may be overcome to enable improved detection.

CHAPTER VII

Development and analysis of heat selected cell sublines

VII. DEVELOPMENT AND ANALYSIS OF HEAT SELECTED CELL SUBLINES

To study the effect of heat stress on GST, I developed a series of cell sublines resistant to heat. It was expected that this should complement the concurrent work on transient heat shock and drug resistance, described in chapter VI. A cell line showing permanent changes in drug resistance as a result of heat treatment would be very useful in studying the regulation of the response. It had been proposed that biochemical changes seen in preneoplasia and drug resistant cell models might be the result of a stress response (Hayes and Wolf, 1988; Lewis *et al*, 1988). The changes seen in drug metabolism as a result of a heat treatment might be part of a general stress response similar to the prokaryotic adaptive response to stress. In *S. typhimurium*, Christman and co-workers have extensively characterised a series of mutants involving the oxidative stress response (Christman *et al*, 1985; Morgan *et al*, 1986; Christman *et al*, 1989). This response appears to consist of a series of genes expressed co-ordinately as members of overlapping regulons. The regulons form sets of genes all induced by the same factor in response to a particular stress, with many of the genes induced by more than one factor. A permanent cell line mimicking the co-ordinate changes normally seen only transiently could be instrumental in characterising the organisation of a similar response in eukaryotes and would be useful in the characterisation of the factors involved in the regulation of the individual genes and any observed co-ordinate response during heat stress.

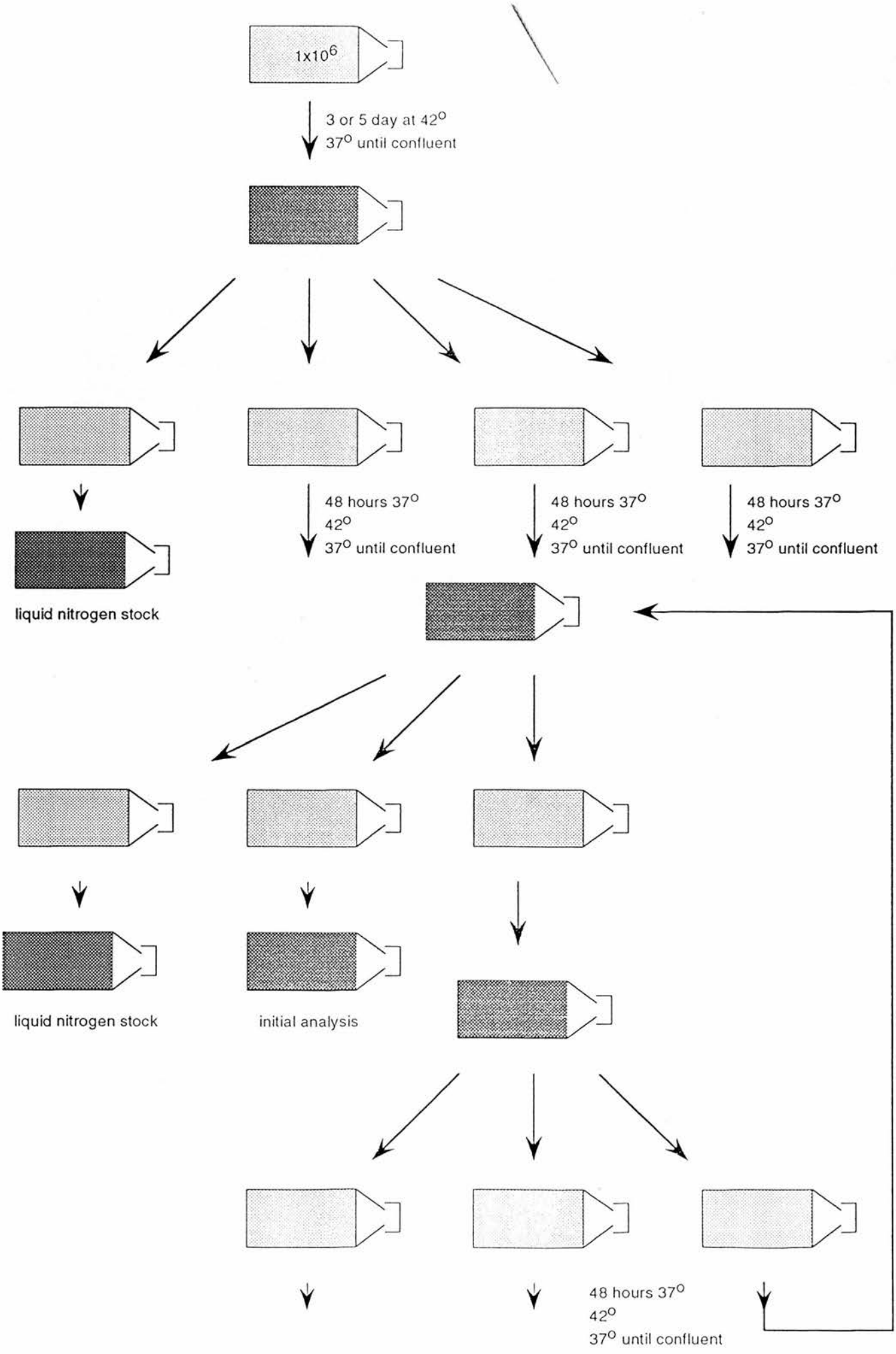
VII.1. Development of heat selected cell lines

The method used to develop the heat resistant cell lines was to expose the cells to sequential heat treatments of increasing exposure time. At each exposure the more heat resistant members of the population survive. Although heat may have some mutagenic effect this selection procedure probably relies on variation that already exists within the wild type population.

The temperature chosen for the heat treatments was 42°C, since at this temperature the fundamental processes essential to the cell, such as transcription and translation are not detrimentally affected. Treatment at this temperature kills cells over a period of several hours to a few days depending on the cell type. The mode of cell death is not known although heat shock does lead to oxidative stress through creation of radicals and superoxide.

Figure 31 describes the protocol used for the development of the cell lines. Approximately $1x10^6$ cells were seeded in $25cm^2$ flasks. These were left 24 hours at 37°C to settle before being exposed to 42°C for 1,2,3 or 5 days. Those flasks whose treatment resulted in more than 90% cell kill, were then left at 37°C until confluent (19-32 days), when they were split into four $25cm^2$ flasks. One of these was grown until confluent, then harvested for storage as liquid nitrogen stocks. The other three flasks of cells were given 48 hours to settle then exposed to longer periods at 42°C. After this second treatment at 42°C, the cells were left at 37°C until they had recovered and grown to confluence. These were then split into three $25cm^2$ flasks. One was used for a liquid nitrogen stock, the second for further

Figure 31: Protocol for the selection of heat resistant NCI H322 cells.


1x10^6
3 or 5 day at 42°
37° until confluent
48 hours 37°
42°
37° until confluent
48 hours 37°
42°
37° until confluent
48 hours 37°
42°
37° until confluent
liquid nitrogen stock
liquid nitrogen stock
initial analysis
48 hours 37°
42°
37° until confluent

analysis and the third to seed further flasks to enable repetition of the procedure with increasing time periods at 42°C. Figure 32 shows the lineage of the heat selected cell sublines and the periods of exposure to 42°C.

After the final heat treatments, the cells were grown for a further 14-20 weeks before any further analysis took place. This was to enable the cells to attain good growth, to prepare frozen stocks and to ensure that any changes observed in the sublines were stable and were not due to a transient alteration in gene expression due to the heat shock.

VII.2. Immunohistochemical analysis of the cell sublines for GST content

I screened 27 cell sublines from the above heat selections for glutathione S-transferases by immunohistochemistry. Cells were seeded into slide flasks, then fixed with methanol and acetone. Antibodies reacting with the three major cytosolic GST families were then used. For several reasons this method can only give a relative estimate of the levels of GSTs present in the cells, as the technique is not quantitative. Firstly, the cytosolic GSTs, being soluble proteins are easily lost from the cells during fixation. Secondly, the product of the enzyme reaction used to detect the antibody binding inhibits the staining reaction. These problems both lead to a reduction in the amount of detected GST compared with the actual amount present. However a reliable comparative estimate can be obtained using densitometry (table 7).

Figure 32: Cell lineage of heat selected NCI H322 sublines.

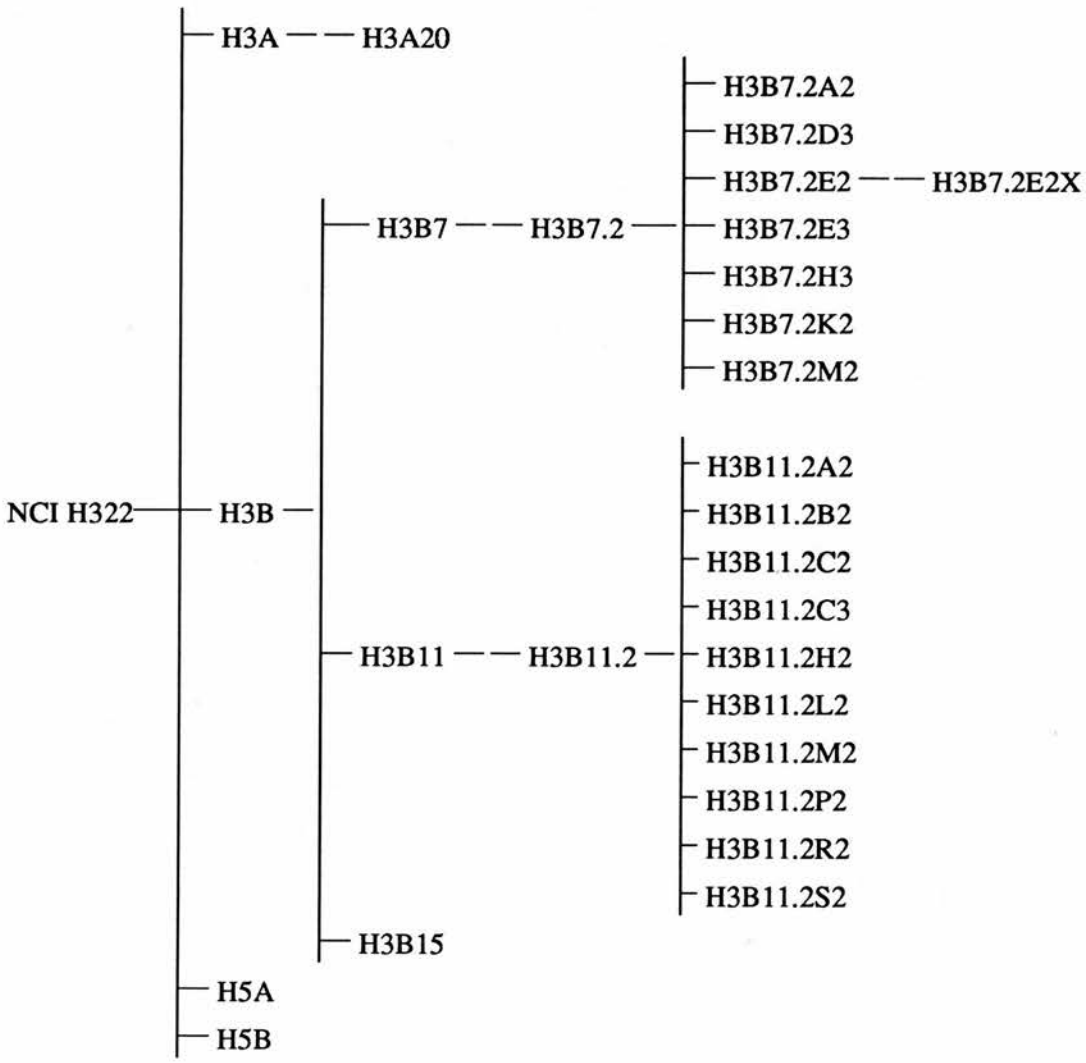


Table 7: Expression of GSTs in heat selected cells measured by immunohistochemistry.

Each heat selected subline was seeded in 9 cm^2 slide flasks and grown until subconfluent. The cells were fixed on the slide using 50% acetone, 50% methanol and stored at -70°C. The fixed cells were then stained for the GSTs using antisera raised against human GST A1, M1 and P1 and a second antibody conjugated to HRP with diaminobenzidine as the substrate as described in chapter II, p40. The intensity of staining was measured using an image analyser as described in figure 3. Each was the average intensity of 100-200 cells.

-	no staining
+/-	less staining than NCI H322
+	approx. NCI H322 levels
++	higher staining levels
+++	
++++	
+++++	highest staining levels

•	Number of selection steps	longest period at 42°C	Alpha	Mu	Pi
NCI H322	0	0d	+	+	+
H3A	1	3 d	+	+	+
H3A20	2	20d	++	+++	+/-
H3B7	2	7d	+	+/-	+/-
H3B7.2	3	14d	+	+/-	+
H3B7.2A2	4	14d	++	++	++
H3B7.2D3	4	21d	+	+++	+
H3B7.2E2	4	14d	+/-	+/-	+/-
H3B7.2E2X	4	14d	+	+++	++++
H3B7.2E3	4	21d	+/-	+	++
H3B7.2H3	4	21d	+	+	+
H3B7.2K2	4	14d	-	+	+++++
H3B7.2M2	4	14d	++	++	++
H3B11	2	11d	-	+	+
H3B11.2	3	14d	-	+/-	+/-
H3B11.2A2	4	14d	+	+/-	+
H3B11.2B2	4	14d	+	+	+
H3B11.2C2	4	14d	+	+	+
H3B11.2C3	4	21d	-	+++	+++++
H3B11.2H2	4	14d	+/-	++	+++
H3B11.2L2	4	14d	+	++	++
H3B11.2M2	4	14d	-	+	+
H3B11.2P2	4	14d	-	+++	++
H3B11.2R2	4	14d	-	-	+
H3B11.2S2	4	14d	-	+/-	+/-
H3B15	2	15d	-	+	++
H5A	1	5d	-	+++	+++
H5B	1	5d	-	+	+

VII.2.1. Alpha class glutathione S-transferase

Variation between the cell sublines is detected although the levels are low. The amount of variation is not great and might be explained by differences in growth conditions (see chapter V), since the sublines were not all plated at the same time, nor was the media changed at the same time relative to fixation. Although differences in Alpha class GST expression can not be ruled out, the differences seen here are not considered significant.

VII.2.2. Mu class glutathione S-transferase

There was no Mu class GST staining detected in the cytosol. The cross reactivity of antisera raised against hepatic Mu class GST (GST-M1) for lung Mu-class GSTs is not good (see chapter V) and there may be Mu class GSTs present which were not detected by the hepatic GST-M1 antibody. Staining was noted in the nuclei of the cells. In 10 of the 27 cell sublines there was an increase in the level of nuclear Mu class staining relative to the wild type cells. Seven of the cell sublines had lower levels and the rest similar levels to the wild type cells. These numbers are not suggestive of a relationship with heat resistance. It has not been established that the nuclear staining in these cells is due to a Mu class GST, although there is some evidence for nuclear GSTs in rat (see chapter VI). The nuclear staining that I detect may be due to a similar protein in human. In chapter VI, a protein of 26K was visualised by Western blot analysis using an antibody raised to a Mu class GST, after cells have been heat shocked (p158).

VII.2.3. Pi class glutathione S-transferase

Immunohistochemistry using an antibody raised against human Pi class GST showed staining in the cytoplasm. The changes in Pi class GST were greater than those noted with the Alpha class GST, whereas with the conditioned media response there were greater changes with the Alpha than with the Pi. This suggested the changes noted with Pi were not due to differences in growth conditions as was suspected with the Alpha class changes. In 11 of the 27 sublines there was a distinct increase in the level of staining relative to the wild type cells. Only 5 of the 27 sublines had lower levels than wild type suggesting there may be a relationship between Pi class GST and heat resistance. Of the 11 with high levels of Pi class GST, only four had higher levels of Mu-class GST, which is well within the range that would be expected by chance. I conclude from this that the cytosolic Pi and nuclear Mu staining protein show no relationship. Proof of a possible correlation, between resistance to the heat treatment in the development of the cell sublines and the expression of Pi class GST, is complicated as many of the sublines share a common ancestry. Several sublines may have derived from a single population within the wild type cell line and perhaps due to random clonal variation both be heat resistant and have increased GST levels.

VII.3: Western blot characterisation of the GST content of three heat selected sublines

In order to further characterise the heat selected cell sublines, three of the sublines were chosen for further analysis. The sublines were chosen to give a representation of the total sublines, one with increased Pi-class

GST, one with increased Mu-class GST and one in which both Pi and Mu-class GSTs were increased.

The H5A subline was derived from a single heat exposure of 5 days. It is thought to be clonal as only a single colony of cells was observed to grow back at 37°C. With immunohistochemical examination H5A showed a slight increase in Alpha class GST and increases in nuclear Mu class GST and cytosolic Pi class GST staining.

H3B7.2K2 was developed through 4 heat treatment steps, the maximum being 14 days at 42°C. The cells showed an increase in Pi class GST staining and no change in Alpha or Mu class GST staining.

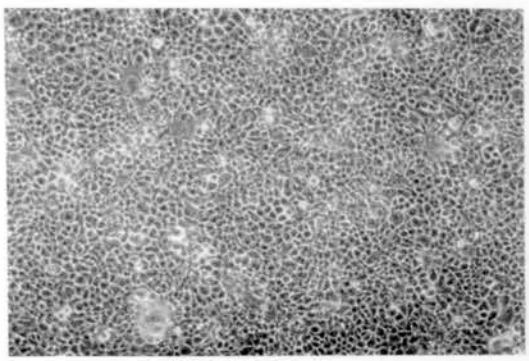
H3B7.2D3 was developed through 4 heat treatment steps, the maximum being 21 days at 42°C. The cells showed no change with Alpha and Pi class GSTs but had a large increase in Mu class GST staining in the nucleus.

These 3 cell sublines and the wild type NCI H322 cells were all brought into culture to enable further corroboration and investigation of the immunohistochemistry results. The H5A and H3B7.2K2 cell sublines both survived the thawing from liquid nitrogen as did the wild type NCI H322 cells. Difficulties occurred with the H3B7.2D3 cells, they did not recover well and many of the cells died, it took several weeks to establish good cell growth.

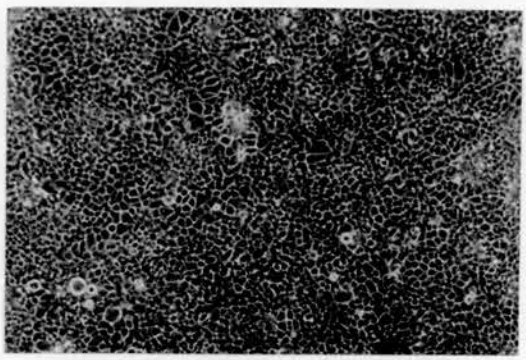
The morphology of the sublines after culture for 14-20 passages was generally similar to that of the NCI H322 wild type cell line (see Figure 33). The wild type cells vary in size and the cell subline sizes were all within the

Figure 33: Heat selected NCI H322 sublines.

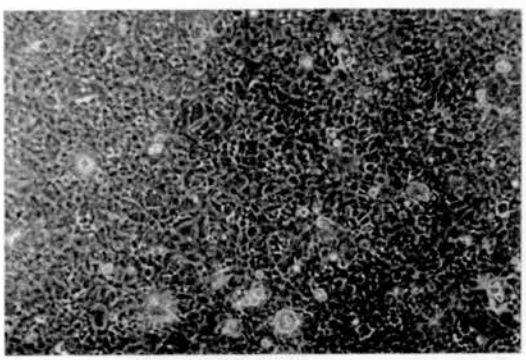
NCI H322 cells and the heat selected cell sublines (H5A, H3B7.2K2 and H3B7.2D3) were seeded in 175 cm^2 flasks at a density of 4 x 10^7 cells. Cells were grown for 2 days before being washed 2 times in PBS and photographed under phase contrast microscopy (x100).



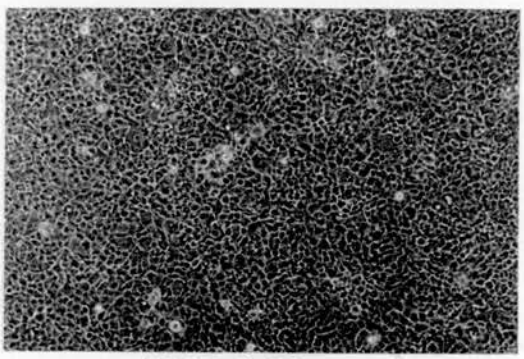
NCI H322



H5A



H387.2K2



H387.2D3

wild type size range. The H3B7.2D3 cells were slightly smaller and the H5A slightly larger than the average NCI H322 wild type cell.

The heat treated cell sublines were grown up to produce enough cells for fractionation. Protein extracts from nuclei and cytosols were assayed for the Alpha, Mu and Pi class GSTs by Western blot analysis (figure 34).

VII.3.1; Alpha class glutathione S-transferase

The Alpha-class GST levels appeared to vary when compared to the wild type. Subline H3B7.2K2 had higher levels and H3B7.2D3 had lower levels than wild type. Since the levels of Alpha class GST in the NCI H322 wild type cell line vary with the culture conditions (see chapter V), the immunohistochemistry data was not considered tenable as the slides had been produced on different days under different conditions. The cells for the Western blots however were grown and harvested under identical conditions, suggesting the difference seen is indeed due to differences in the genotype of the cell sublines.

VII.3.2. Mu class glutathione S-transferase

No Mu class GST was detected in the nucleus of the heat selected cell sublines with any of the different Mu-class antisera. In the cytosols of the heat treated cell lines analysed with the Mu-class antisera M1-JN and M1-SL, GST-M1 was not detected. There was however another protein detected within the cytosol which is distinct from GST-M1. This protein migrated with a higher mobility, comparable to the non polymorphic band detected in human liver. The different cell sublines have varying levels of this protein; with subline H3B7.2K2 having significantly higher levels than

Figure 34: Expression of GSTs in heat selected NCI H322 cells measured by Western blot.

NCI H322 cells and the heat selected cell sublines (H5A, H3B7.2K2 and H3B7.2D3) were seeded in 175 cm^2 flasks at a density of 4 x 10^7 cells. Cells were fed on the following day and harvested on the next. Cytosolic and nuclear protein was prepared. Methods are as described in chapter II. Cytosol (20μg) [C] and nuclear (40μg) [N] samples were subjected to SDS-PAGE, transferred to nitrocellulose and Western blotted with antisera raised against Alpha (A1-NM) Mu (M1-JN) and Pi (P1-λ) class GSTs. Human liver cytosols from two people, one of whom was null for GST-M1 (lv XII) were run alongside (lv X and lv XII). Also GST standards, human Alpha GST (A1), mouse Mu class GST (Yb_1) and mouse Pi class GST (Yf), were run alongside the samples. Unlabelled tracks are not relevant to this experiment.

GST-A1
(NM)
- A1 (100ng)
- A1 (20ng)
- H3B7.2D3 [N]
- H3B7.2K2 [N]
-
-H5A [N]
- NCI H322 [N]
- H3B7.2D3 [C]
- H3B7.2K2 [C]
-
-H5A [C]
- NCI H322 [C]
- lv XII
- lv X
GST-M1
(JN)
- Yb1 (100ng)
-
- H3B7.2D3 [N]
- H3B7.2K2 [N]
-
- H5A [N]
- NCI H322 [N]
- H3B7.2D3 [C]
- H3B7.2K2 [C]
-
- H5A [C]
- NCI H322 [C]
- lv XII
- lv X
GST-P1
(λ)
- H3B7.2D3 [N]
- H3B7.2D3 [N]
- H3B7.2K2 [N]
-
-H5A [N]
- NCI H322 [N]
-
- H3B7.2D3 [C]
- H3B7.2D3 [C]
- H3B7.2K2 [C]
-
-H5A [C]
- NCI H322 [C]
- lv X
- Yf (20ng)

the wild type cell line. This may also be the same protein detected when induced in the wild type cells when underfed or when grown to high confluence levels (p106). There was an invariant band present with the same mobility as GST-M1. However it was not thought to represent GST-M1 since it was not detected by any of the other antibodies raised against it. It could possibly be either a related GST or a background band specific to the M1-JN antisera.

None of the antibodies raised against GST-M1, including M1-MR (the antibody used in the immunohistochemistry) and M1-SH, (the antisera which detected the Mu class GST in the nucleus of heat shocked cells [chapter VI, p133]), show any GST present in the nucleus. This appears to contradict the findings of the immunohistochemistry. It is quite plausible that the antibodies may only detect the antigen when in a particular conformational state as discussed in chapter VI. The M1-MR antibody may detect the Mu class antigen present in the nucleus, when the antigen is in a different conformational state found with methanol/acetone fixation. The same antigen may not be recognised on a Western blot as the protein is in a denatured state. This problem could probably be investigated further by either direct immunoprecipitation or Western blot analysis of a non denaturing gel.

VII.3.3. Pi class glutathione S-transferase

On Western blot analysis the levels of Pi-class GST varied and corresponded well with the immunohistochemistry results (figure 34 and table 7). Both the sublines H3B7.2K2 and H5A had levels higher than the wild type; H3B7.2K2 showed the highest levels. Subline H3B7.2D3 has Pi

class GST levels very similar to the wild type cell line. The variation was confirmed by repetition.

VII.4. Northern analysis of Pi class GST mRNA levels

To establish the basis for the increased expression of GST within the heat selected cell lines, RNA was prepared from each of the cell lines. This was then subjected to Northern analysis with probes for Alpha (A1), Pi (P1) and Mu (M1) class GSTs. Figure 35 shows that the heat selected lines H5A and H3B7.2K2 had Pi class mRNA levels higher than the wild type, H3B7.2K2 had the highest levels. Northern analysis with both the Alpha class GST and a Mu class GST genomic fragment failed to detect any mRNA although detecting mRNAs from human liver.

VII.5. Southern Analysis of heat selected sublines

Southern blot analyses (figure 36) showed no obvious changes in gene copy number which could be responsible for changes in Alpha and Mu class GST protein levels. Southern analysis with the GST-P1 genomic fragment showed that the fragment contained a DNA repeat element rendering it unsuitable for use against genomic DNA although it worked well against mRNA (see figure 35).

VII.6. Drug sensitivities of the heat selected sublines

In order to establish if the changes seen in the heat treated cell sublines could have any effect on the drug resistance properties of the cell sublines, cytotoxic assays, involving several drugs often used in cancer chemotherapy, were carried out.

Figure 35: Northern blot analysis of the heat selected cell sublines.

Total RNA was prepared from wild type and heat selected sublines of NCI H322 (H5A, H3B7.2K2 and H3B7.2D3). Cell lines were seeded at 2 x 10^7 and grown for 2 days. 10 μg each RNA was electrophoresed alongside RNAs from human (hum lv) and rat liver (rat lv) and rat lung (lg). Equivalent loading was confirmed by ethidium bromide staining. Fractionated RNA was transferred to Hybond-N before Northern blot analysis with a human GST-P1 genomic DNA fragment.

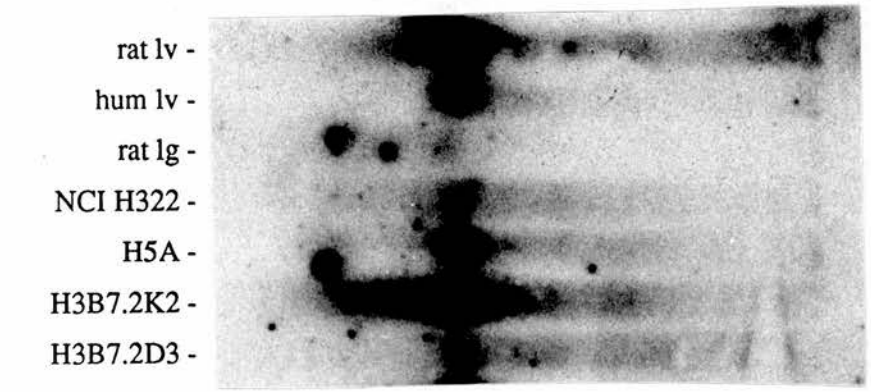
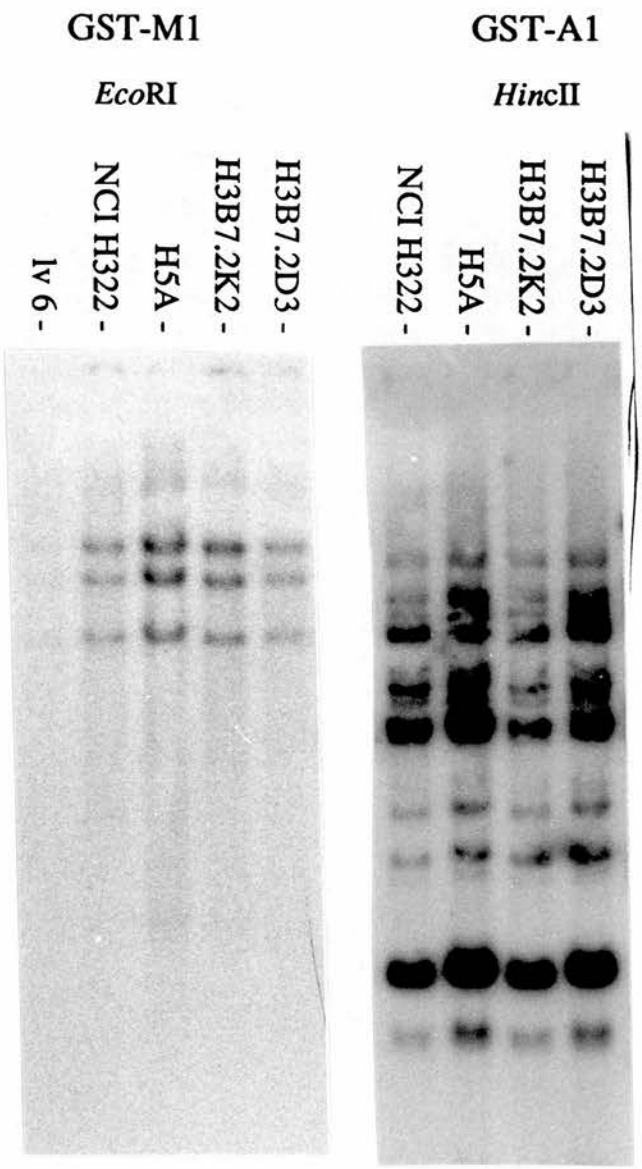

rat lv -
hum lv -
rat lg -
NCI H322 -
H5A -
H3B7.2K2 -
H3B7.2D3 -

Figure 36: Southern blot analysis of the heat selected cell sublines

Genomic DNA was prepared from NCI H322 cells and the heat resistant cell sublines, H5A, H3B7.2K2 and H3B7.2D3, as described in chapter II, p 44. DNA digested with the DNA restriction enzymes *Eco* RI and *Hinc* II was fractionated on a 0.8% agarose gel and transferred to Hybond-N before Southern analysis with ^{32}P-labelled fragments corresponding to a human Mu class GST genomic sequence from J.Taylor (jt14) and the human GST-A1 cDNA. Marker is 1 kb marker from BRL (M).


GST-M1
EcoRI
lv 6 -
NCI H322 -
H5A -
H3B7.2K2 -
H3B7.2D3 -
GST-A1
HincII
NCI H322 -
H5A -
H3B7.2K2 -
H3B7.2D3 -

A required characteristic in the comparison of GST levels between the sublines and the wild type cells is that they have similar growth properties. Many of the drugs used in chemotherapy target rapidly growing cells. If the cell sublines have markedly different generation times then it would be unclear, if a difference in the drug sensitivity of a cell subline is a result of a change in gene expression or is the result of the increased effectiveness of the drug on more rapidly dividing cells.

A similar problem exists with the plating efficiency of the cells. The effective intracellular concentration of a drug within a small volume of a flask may be quite markedly affected by the number of cells within the flask. For example. if the cells detoxify the drug, the higher the number of cells present, the shorter the effective exposure time to the drug. The simplest assay to use for measuring the growth characteristics of the cell lines is the MTT microtitre plate assay. This assay is based on the fact that a viable cell will metabolise the soluble yellow compound MTT to produce a deep purple insoluble dye. A known number of cells from each cell line are plated and grown in 96 well microtitre plates. After 4 or 5 days the MTT is added and left on the cells for 4-6 hours. The amount of compound metabolised to give the insoluble purple dye is proportional to the number of viable cells in the well. The purple dye crystallises and sinks to the bottom of the well and can then be measured by aspirating off the media and dissolving the MTT crystals in DMSO. The absorbance can then be measured with an ELISA reader.

To compare the growth rates and plating efficiencies of the cell sublines and the wild type NCI H322 cells, the growth of the cells on microtitre plates were compared. Varying numbers from each cell subline

were plated out on 96 well plates. After four days the MTT assay was performed and the results are shown in figure 37. The cell sublines seemed to plate out and grow similarly, especially up to $5x10^4$ cells, well above the numbers normally used in the assay. Above this density the platings do vary even within the results for one cell subline. Presumably this is because as the monolayers became confluent, the cells continued to divide and sometimes the monolayer peeled off. Above a certain density the MTT crystals also tended to float in the media rather than settle on the monolayer at the bottom of the well, which made it very difficult to aspirate off the media.

The appropriate concentration range of each drug to use in the assays was established in a preliminary MTT assay. Dilutions of a 1 mM solution in media of each drug were added in triplicate to $2x10^4$ cells in a well in 100µl media. After 2 hours the media containing the drug was removed and replaced with fresh media. Five days later, the number of viable cells in each well was estimated using the MTT assay. The results are shown in figure 38. From this experiment, suitable dilutions of each drug were chosen to enable testing for variation in the cytotoxicity of the drugs in each of the cell lines.

Different drugs vary quite significantly in the slope of their cytotoxicity curves. This makes comparison of drug resistance between two cell lines, by measuring the fold difference in the concentration of the drug at which 50% cytotoxicity occurs, very difficult. For example (figure 39), a cell line displays a 2 fold increase in resistance to drug A and a 16-fold increase to drug B. It would be reasonable therefore to assume that the change in resistance to drug B is much more significant. If one examines the

Figure 37: Comparison of the growth characteristics of heat selected NCI H322 cells

Two fold serial dilutions of each NCI H322 cell subline were seeded in 96 well plates. Cell numbers ranged from 40 cells/well to 6.4×10^5 cells/well. The media was changed alternate days and after 4 days, the numbers of cells in each well was then assayed for using MTT. The relative number of viable cells after 4 days was then plotted against the initial seeding numbers for each cell subline and the progenitor NCI H322 cells.

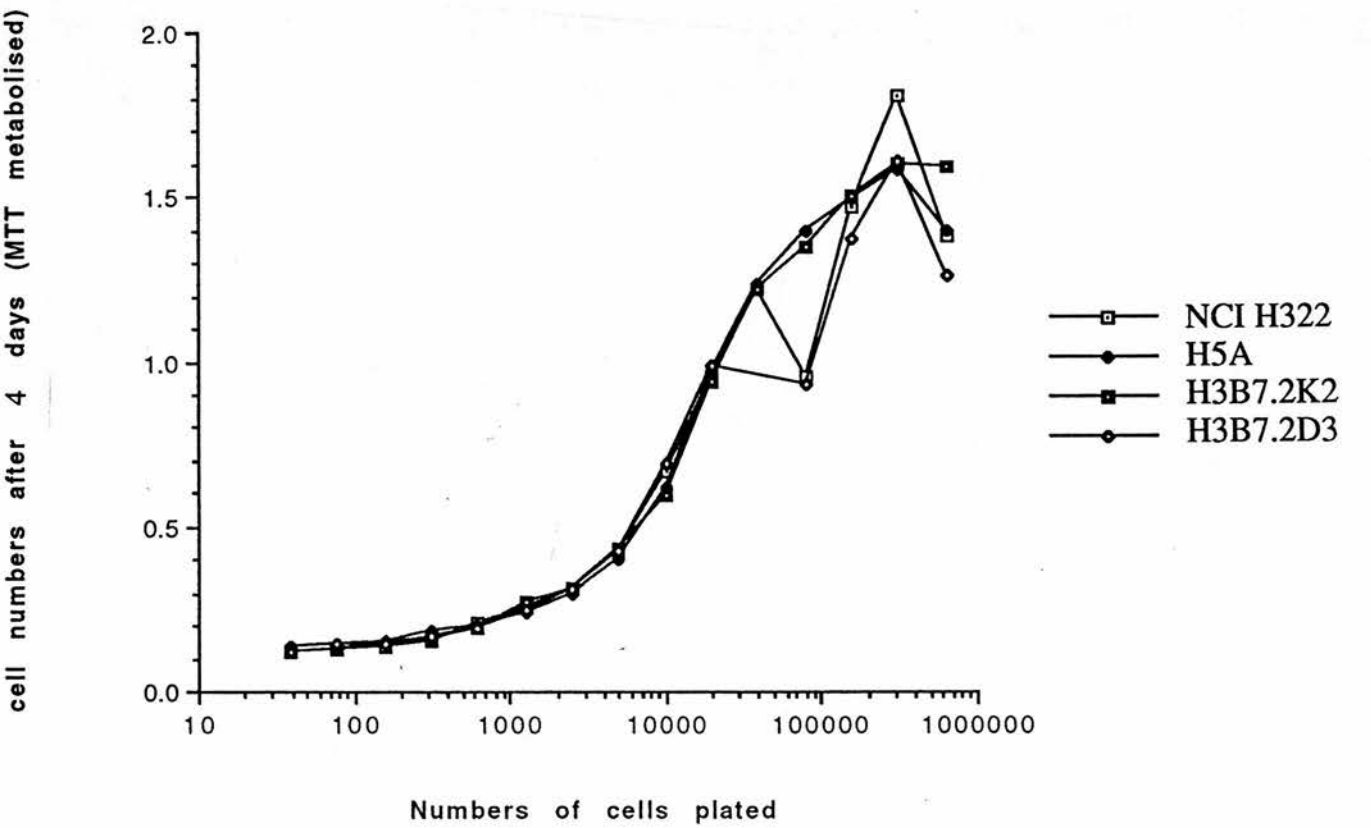

cell numbers after 4 days (MTT metabolised)
2.0
1.5
1.0
0.5
0.0
10
100
1000
10000
100000
1000000
Numbers of cells plated
NCI H322
H5A
H3B7.2K2
H3B7.2D3

Figure 38: Estimation of drug concentrations to use in the heat treated cell subline cytotoxicity assays.

$2x10^4$ NCI H322 cells were seeded in 96 well microtitre plates in 100 ml media. After 2 days, dilutions of a 1 mM solution of each drug were made up in media and added in triplicate to the cells. After 2 hours the media containing the drug was removed and replaced with fresh media. Five days later, the number of viable cells in each well was estimated using the MTT assay.

Drugs used were as follows:

CDNB	1-chloro-2,4-dinitrobenzene
EA	ethacrynic acid
Adr	adriamycin
BCNU	1,3-bis(chloroethyl)-1-nitrosourea
tSO	*trans*-stilbene oxide
Men	menadione

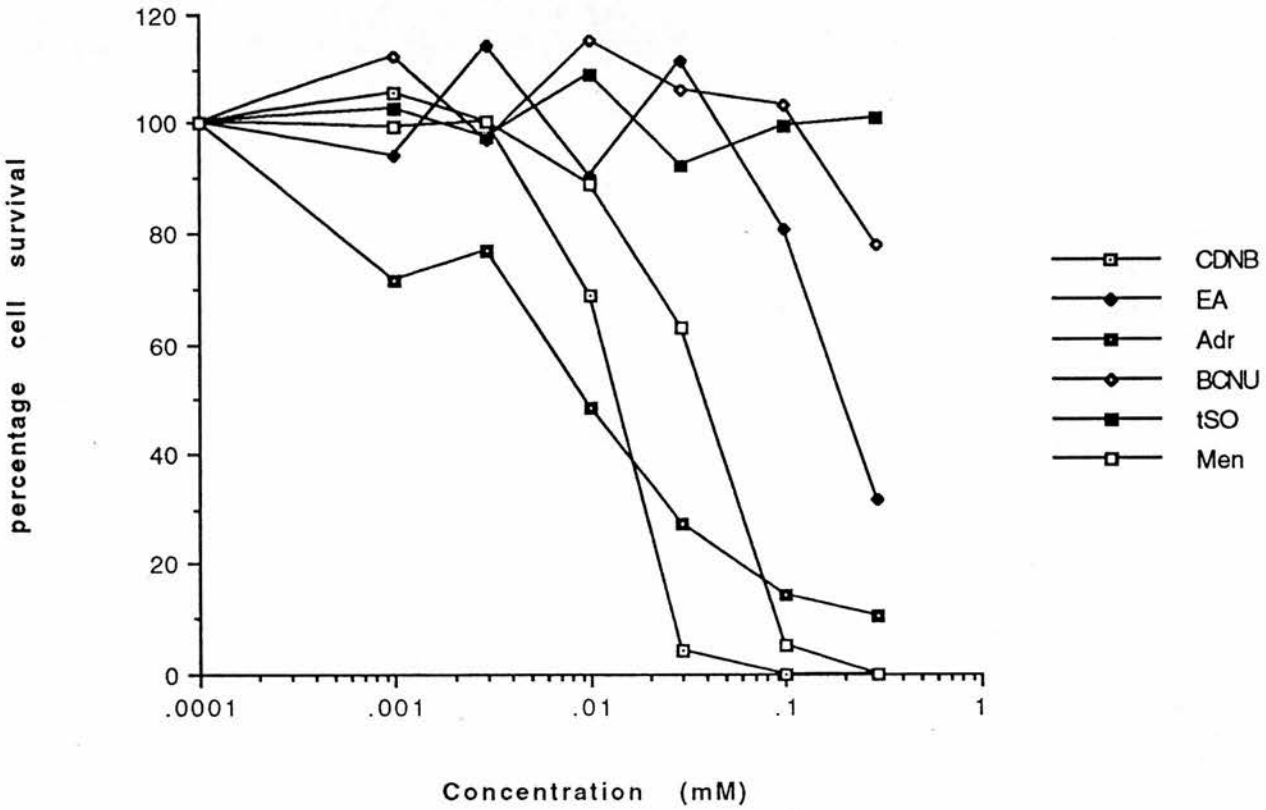
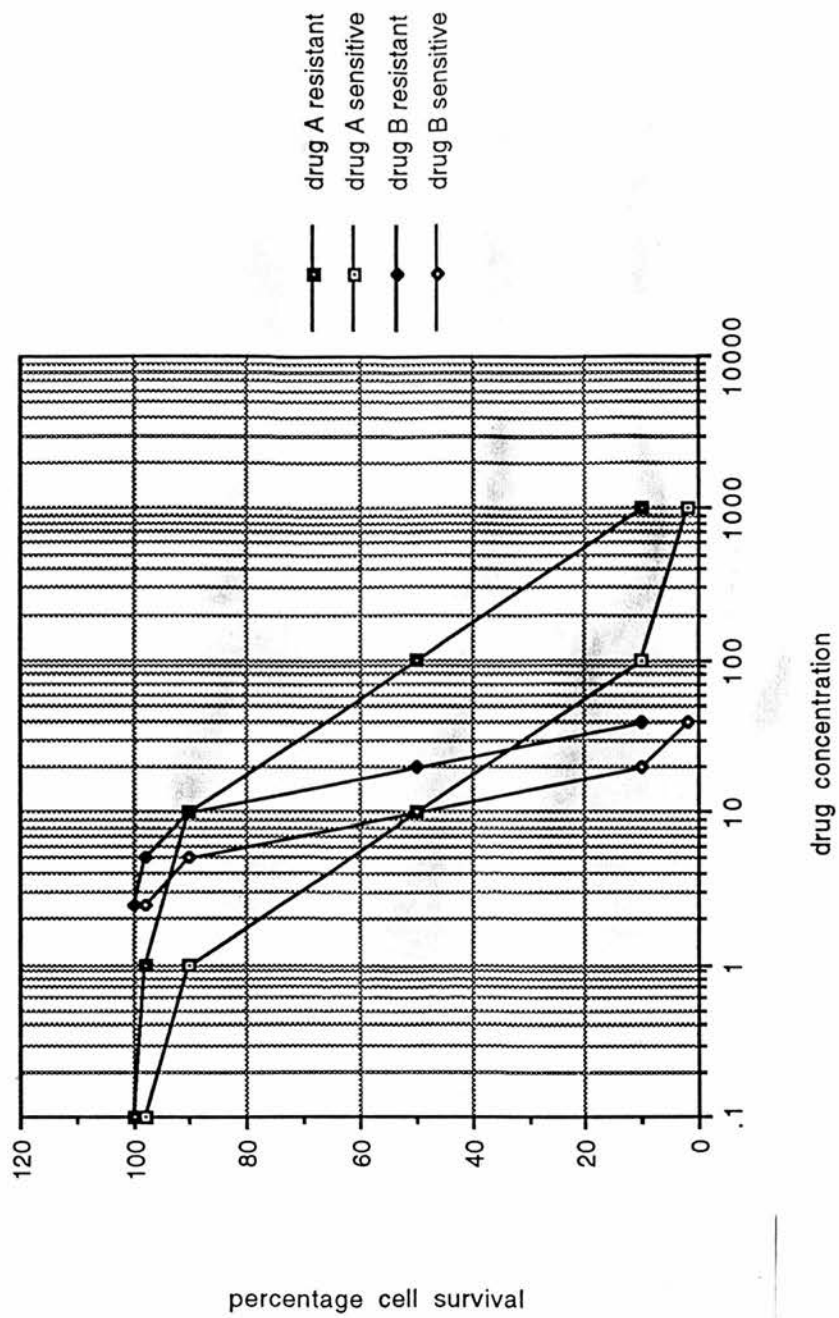

percentage cell survival
120
100
80
60
40
20
0
.0001
.001
.01
.1
1
Concentration (mM)
CDNB
EA
Adr
BCNU
tSO
Men

Figure 39: Difference between measuring fold resistance and cell survival.

A resistant cell line displays a 2 fold increase in resistance to drug A and a 16-fold increase to drug B. Due to the difference in the slope of the cytotoxic curve, both drugs have the same percentage cell survival variation, between the resistant and sensitive cells lines; i.e. at the concentration which kills 50% of the resistant cells, 10% of the sensitive cells survive.


drug A resistant
drug A sensitive
drug B resistant
drug B sensitive
120
100
80
60
40
20
0
.1
1
10
100
1000
10000
percentage cell survival
drug concentration

data in terms of the percentage of cells surviving, it can be seen for both drugs, that at the concentration which kills 50% of the resistant cells, 10% of the sensitive cells survive. In terms of the percentage of cells surviving, both changes in resistance have equal significance. For this reason, although changes in drug resistance have been stated as fold resistance in agreement with the literature, the data have also been expressed as the percentage of cells surviving a certain concentration of drug. I feel that the percentage cell survival gives a clearer description of the data than the measurement of fold resistance, especially when relating the data to drug resistance in tumours, as it is the percentage of cells surviving within a tumour after a course of treatment which determines whether a tumour will reappear.

The results of the cytotoxicity assays are shown in figure 40 and table 8. The drug sensitivities of the cells to ethacrynic acid did not show any significant changes. Both H5A and H3B7.2K2 appeared more resistant to CDNB. Subline H3B7.2D3 was apparently slightly more sensitive to CDNB than the wild type cell line. Although the differences noted were not great, they were repeatable. Subline H3B7.2K2 showed an increased resistance to adriamycin. Cell subline H5A showed an increased resistance to menadione. This represents only an estimated 1.6 fold increase in LD_{50}, but at a concentration of 5.9×10^{-5}M, a concentration at which only 33% of the wild type NCI H322 survive, over 80% of the H5A subline survive the 2 hr treatment.

VII.7. Heat sensitivity of the sublines

An attempt was made to measure the heat resistance of the cell lines at 42°C, the temperature used to develop the cell lines. This proved

Figure 40: Estimation of drug sensitivities of the heat selected cell sublines using the MTT assay.

Cytotoxicity assays were carried out as described in chapter II. 2 x 10^4 cells/well were seeded in 96 well plates. After 24 hours cells were treated with serial dilutions of the following drugs; [A] CDNB and adriamycin (Adr); [B] ethacrynic acid (EA) and menadione (Men). After 2 hours the drugs were washed off and the cells fed with fresh media. Four or five days following the treatment the numbers of viable cells were measured using the MTT assay. The amount of MTT metabolised was then plotted against the drug concentration. Each data point is the average from 6 wells.

Figure 40 A

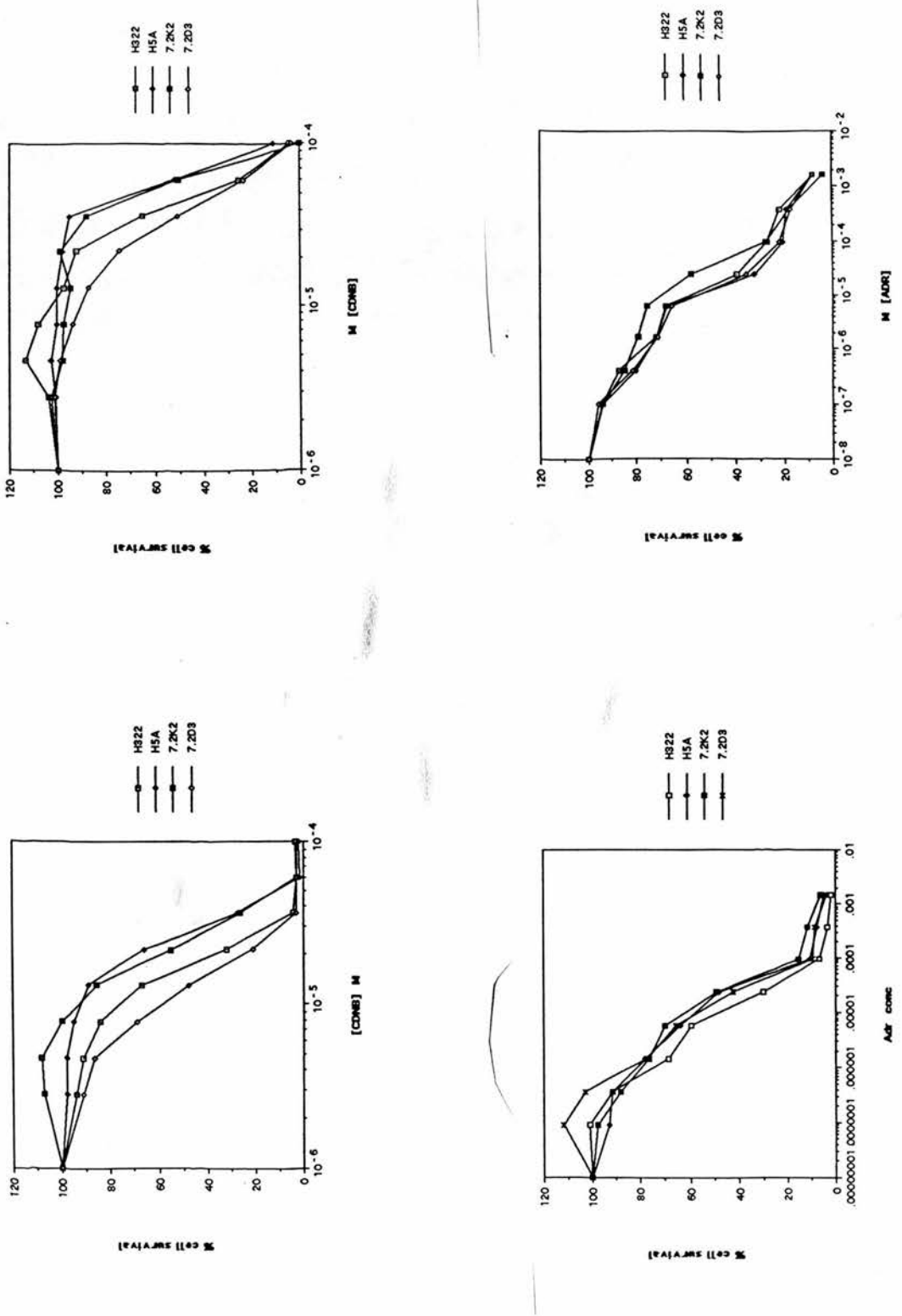
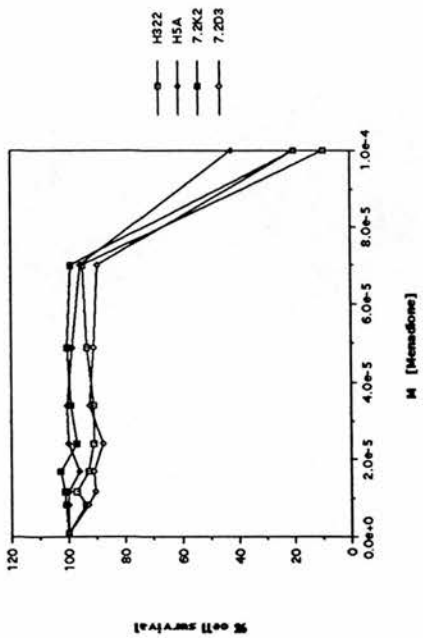
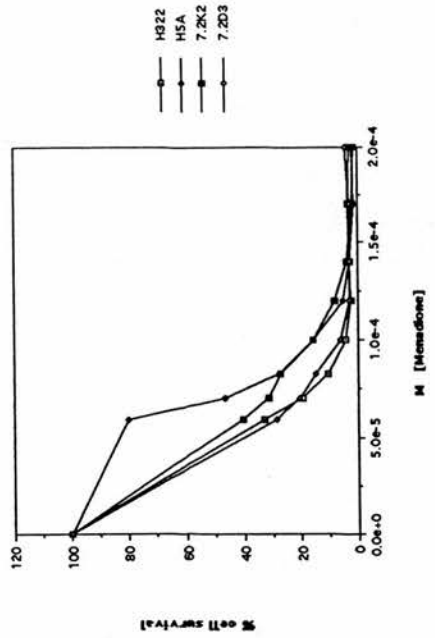
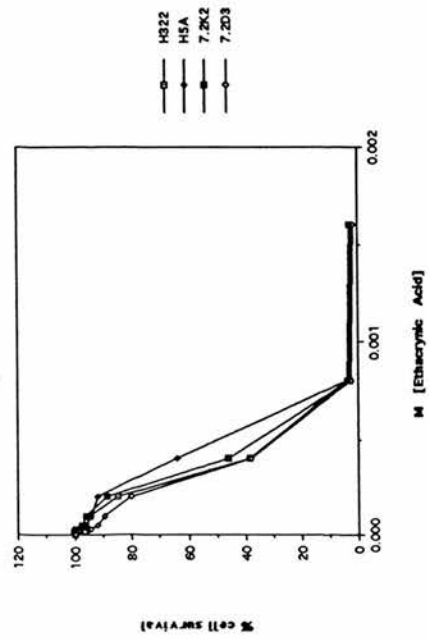


Figure 40 B


H322
H5A
7.2K2
7.2D3
0.0e+0
2.0e-5
4.0e-5
6.0e-5
8.0e-5
1.0e-4
M [Menadione]
0
20
40
60
80
100
120
% cell survival


H322
H5A
7.2K2
7.2D3
0.0e+0
5.0e-5
1.0e-4
1.5e-4
2.0e-4
M [Menadione]
0
20
40
60
80
100
120
% cell survival


H322
H5A
7.2K2
7.2D3
0.000
0.001
0.002
M [Ethacrynic Acid]
0
20
40
60
80
100
120
% cell survival

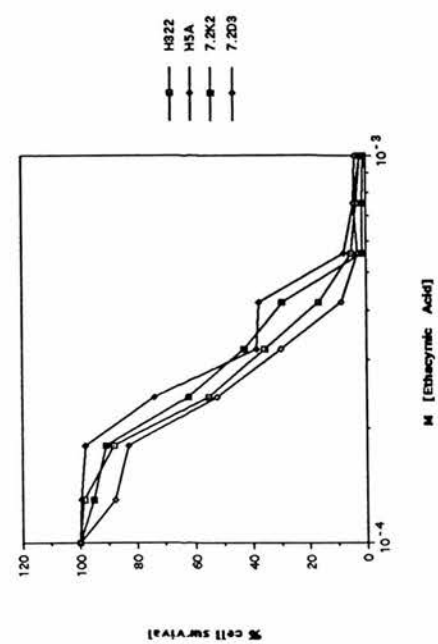

H322
H5A
7.2K2
7.2D3
10^-4
10^-3
M [Ethacrynic Acid]
0
20
40
60
80
100
120
% cell survival

Table 8: Drug sensitivities of the heat selected sublines

The concentration of drug which killed 50% of the cells (LD_{50}) was taken from the cytotoxicity curves of the heat selected cell sublines (figure 40) and the fold resistance calculated (table A). Table B shows the % cell survival at specific drug concentrations. Data for each drug were taken from 2 separate experiments.

	LD50				fold resistance		
A	H322	H5A	7.2K2	7.2D3	H5A	7.2K2	7.2D3
CDNB	17μM	27μM	24μM	12μM	1.6	1.4	0.73
	45μM	61μM	61μM	36μM	1.4	1.4	0.76
Adr	18μM	15μM	44μM	16μM	0.8	2.4	0.9
	8.3μM	16μM	52μM	13μM	1.9	6.2	1.5
EA	350μM	490μM	380μM	340μM	1.4	1.1	1.0
	250μM	360μM	290μM	240μM	1.4	1.2	1.0
Men	86μM	95μM	89μM	87μM	1.1	1.0	1.0
	44μM	69μM	50μM	41μM	1.6	1.1	0.9

B	concentration	H322	H5A	7.2K2	7.2D3
CDNB	22 μM	32%	66%	54%	22%
	60 μM	25%	51%	49%	23%
Adr	25 μM	38%	33%	58%	36%
	25 μM	27%	35%	74%	24%
EA	400 μM	38%	64%	45%	38%
	240 μM	54%	73%	62%	52%
Men	100 μM	8%	42%	20%	20%
	59 μM	31%	79%	39%	28%

difficult, due to the long exposure times necessary, which introduced too much error into the procedure. Instead, the resistance of the cell lines to 45°C was measured as this required much shorter exposure times.

The three cell sublines and the wild type NCI H322 cells were plated out at 2 x 10^4 cells per well in microtitre plates. The plates were then exposed for varying lengths of time at 45°C, before being left to recover and grow at 37°C for 4 days. An MTT assay was then carried out and the results are shown in table 9. Subline H3B7.2K2 shows better survival than the wild type cell line. However, sublines H5A and H3B7.2D3 did not. H5A may not be expected to show any resistance to heat as it was selected with just a single step treatment.

On the other hand, sublines H3B7.2K2 and H3B7.2D3 were developed with the longer heat exposure times of two and three weeks at 42°C respectively. In bringing subline H3B7.2D3 back into culture from liquid nitrogen storage however, only a small fraction of cells survived, perhaps less than 1%. This may have resulted in loss of phenotype, either the cells reverted to wild type phenotype or as the sublines were not clonal, only a non resistant population survived.

In the future it would be important to return to the frozen stocks to try to recover the H3B7.2D3 cell subline or alternatively to use another subline which showed high levels of the nuclear Mu class GST staining with immunohistochemical analysis. This may enable identification of the nuclear protein.

Table 9 Heat sensitivity of the heat selected sublines as measured by MTT assay.

A comparison of the heat sensitivity of the NCI H322 heat selected cell sublines was carried out. In two separate experiments 2 x 10^4 cells/well were seeded in 96 well plates. After 24 hours at 37°C plates were then placed at 45°C for the specified time (240 or 340 minutes), then returned to 37°C for 4 days, after which the numbers of viable cells were estimated using the MTT assay. The MTT metabolised by the cells treated at 45°C was expressed as a percentage of the untreated cells.

	experiment 1		experiment 2	
minutes at 45°C	240 min	340 min	240 min	340 min
NCI H322	22%	3%	35%	10%
H5A	19%	2%	39%	9%
H3B7.2K2	39%	18%	53%	32%
H3B7.2D3	9%	1%	30%	4%

VII.8. Discussion

Twenty seven cell sublines were obtained after various exposures to 42°C heat. Immunohistochemical analysis suggest 11 of the sublines had increased Pi class GST and 10 had increased staining in the nucleus with GST-M1 antisera. No correlation with Pi and Mu class GSTs was seen. Small differences in Alpha class GST were noted but these might be explained by differences in the culture conditions. After this initial screening three sublines were chosen for further study. The levels of GSTs were estimated by western blot analysis and the drug sensitivities measured. This is summarised in table 9. Subline H3B7.2K2 shows higher levels of Pi class GST and a Mu class GST detected in the cytosol with M1-JN antisera with a faster mobility than GST-M1 (μ). This subline shows slight but repeatable increases in resistance to adriamycin and CDNB, as well as showing some thermotolerance. H5A has higher levels of the Pi class GST and shows increases in resistance to menadione as well as a slight increase in resistance to CDNB. It does not show any thermotolerance, most probably because it was derived from a single selection step. The subline H3B7.2D3, which was thought to have been lost when recovered from frozen stocks, showed no changes in GST; interestingly it does not show any heat or drug resistance.

Identification of the importance of an isoenzyme in a particular reaction is difficult due to the overlapping substrate specificities.

Table 10 Summary of immunological and cytotoxicity data

Summary of the data from immunohistochemical (IHC) and Western blot (W) analyses of Alpha, Mu and Pi class GSTs and the cytotoxicity assays for CDNB, adriamycin, ethacrynic acid and menadione, in the heat selected NCI H322 cell variants.

	IHC staining		Western blot		Cytotoxicity assay
-	no staining	-	no protein		
+/-	less than wt.	+/-		+/-	less resistant than wt.
+	wt. levels	+	wt. levels	+	no change
++		++	higher protein levels	++	more resistant than wt.
+++		+++	highest protein levels	+++	
++++				++++	highest resistance
+++++	strongest staining				

	NCI H322	H5A	H3B7.2K2	H3B7.2D3
IHC Alpha	+	-	-	+
W Alpha	+	++	+++	+/-
IHC Mu	+	+++	+	+++
W Mu nuclear	-	-	-	-
W Mu cytosol	+/-	+/-	++	+/-
IHC Pi	+	+++	+++++	+
W Pi	+	++	+++	+/-
CDNB	+	+++	+++	+/-
Adriamycin	+	+	++++	+
Ethacrynic	+	++	++	+
Menadione	+	+++	+	+

Both H5A and H3B7.2K2 showed increased resistance to CDNB. This is the general substrate used to detect the glutathione S-transferases although there is marked variation in the activity of individual subunits for CDNB (Mannervik and Danielson, 1988). The alkylating agents are not transported by P-gp and as CDNB is a good substrate for GST it is likely that overexpression of GST is a major mechanism for resistance to CDNB. A NCI H322 cell line was selected for resistance to CDNB and was found to overexpress Alpha class GST (C.Wareing, personal commun.). H3B7.2K2 has higher levels of all three cytosolic GST classes but does not appear to have higher resistance than H5A. There are a number of reasons why this is not surprising. Overexpression of GST is probably not the only mechanism of resistance for CDNB and it is possible that not all GST subunits are being detected by the antisera used in the Western analysis. The H5A cell line stained strongly for Mu class GST in the nucleus, although this was not detected on Western analysis. It is possible that this putative subunit is responsible for the CDNB activity noted in the H5A cells.

The heat selected cell subline H3B7.2K2 showed increased resistance to Adriamycin. Both human GST-A1 and P1, as well as rat Ya and Yb_1 have been shown to confer resistance to adriamycin (Black *et al*, 1990; Pukalski and Fahl, 1990; Nakagawa *et al*, , 1990). Alpha, Mu and Pi class GST subunits are all elevated in the H3B7.2K2 cell line relative to the progenitor cell line, it is therefore likely the increased resistance is related to the overexpression of GST. Use of inhibitors such as ethacrynic acid or the development of revertants would help to establish this.

Both H5A and H3B7.2K2 showed a slight but repeatable resistance to ethacrynic acid whose metabolism has been associated with GST P1,

GST-A1 (Leyland-Jones *et al*, 1991) and GST-A2 (Townsend *et al*, 1992). These two cell lines were shown to have increases in Alpha and Pi class GSTs suggesting this may be the mechanism for the slight increases in resistance seen.

H5A shows resistance to menadione which is thought to cause oxidative stress. No correlation with the cytosolic GST subunits can be made as H3B7.2K2 shows no increased menadione resistance. This is corroborated by data showing no changes in GST following a transient menadione exposure (K. Vallis, personal commun.).

Huot *et al* (1991) describe making a transfectant of hsp 27, which confers heat resistance to cells. This protein can also confer resistance to adriamycin. It is possible that the heat selected NCI H322 cells may have increased levels of hsp 27. This could explain the increased resistance to adriamycin in the NCI H322 heat selected cells. An antibody to hsp27 could perhaps be obtained to establish if this is likely.

It is difficult to establish conclusively the relationship between an observed change in protein level and the observed resistance in a cell line. One method of doing this is by using revertants and associating loss of the resistance phenotype with the loss of the protein.

CHAPTER VIII

Summary and concluding remarks

VIII. SUMMARY AND CONCLUDING REMARKS

As a growing body of evidence demonstrates that GSTs are involved in drug resistance, it is important to understand the regulation of the levels of these proteins, and the reasons behind the increased levels in drug resistance. Such an understanding will be important in attempts to prevent drug resistance occurring and to reverse resistance once it occurs during chemotherapy. It had been proposed that increased levels of GSTs may be part of a general stress response where exposure to a single stress may lead to expression of a variety of other proteins, not all required by the inducing stress but leading to resistance to other stresses. This had been proposed to explain the preneoplasia and drug priming models where cross resistance develops to a variety of environmental stresses. A permanent alteration in the regulation of this response in a tumour cell may result in constitutively expressed drug resistance (Hayes and Wolf, 1988). The specific aims were to investigate the stress inducibility of the GSTs and to investigate the possibility of developing a model system to study this regulation.

A potential model developed in the laboratory was a chlorambucil resistant CHO cell line. Similar changes in Alpha class GST and γGT protein levels had been detected both in these cells and in an *in vivo* drug priming model. I have shown that the Alpha class GST encoding DNA is amplified and the mRNA overexpressed in the chlorambucil resistant cell line. This demonstrates gene amplification as a potential mechanism for drug resistance due to GST *in vivo*. The γGT encoding DNA is not amplified indicating the cause of the overexpression of the two proteins is different. In addition, in an oxygen resistant CHO cell line Alpha class GST protein and mRNA has also been shown to be overexpressed. Transient

exposure, of the wild type CHO cells, to 98% oxygen also induced a similar Alpha class GST. This may be indicative of a role for this protein in oxidative stress as well as in drug resistance.

A heat shocked lung tumour cell line showed some changes in the levels of Pi and Mu class GSTs but not Alpha class GST. Pi class GST was detected to increase 2-4 fold following heat shock of a lung tumour cell line at both 45°C and 42°C. The Pi class mRNA levels during heat shock decreased, suggesting the increase seen in protein may have been due to an increase in protein stability perhaps relative to other cellular proteins. Perhaps more significantly a putative Mu class GST subunit was identified in the nucleus of heat shocked cells. The induction of this nuclear protein was shown by two independent antibody preparations. The immunohistochemistry also showed increases in nuclear staining with a Mu class GST antisera in the CHO cell line exposed to high levels of oxygen. The nuclear Mu class GST staining has not been further characterised due to the problems with detection on Western blotting. This putative nuclear Mu class GST in the heat shocked lung cells may represent a subunit induced by both heat and oxidative stress. Future studies would be required to both identify this protein and to establish its pattern of expression in stress.

In the models studied there did not appear to be many similarities in the GST changes noted. The CHO cells exposed to oxidative stress showed changes in the Alpha class GSTs; whereas the human lung cells when exposed to heat shock showed changes in Pi class GST. These studies do not suggest regulation of the cytosolic GSTs as part of a general response to stress. Although a general stress response could be masked by the species

and tissue differences in the isoenzyme patterns, it seems likely that the changes noted may be specific to the inducer stress.

Inconsistencies in the data from the preliminary induction experiments led to the investigation of the effect of growth conditions on GST levels. Isoenzymes from three classes of GST were found to be elevated by increased confluence and a low frequency of feeding. This response was found to be mediated, at least in part, through conditioning of the culture media. Preliminary analysis suggests the factor responsible has a molecular weight of less than 14 kD but it is not due to an alteration in the pH or redox balance of the media. The regulation of this response was studied and the data suggest that the mechanism is not transcriptional but due to a change in protein stability or translational efficiency (p114). This is another level of regulation through which the GST isoenzyme levels may be elevated, adding to those already described; transcriptional activation, gene amplification and mRNA stability (Morrow *et al*, 1992).

Even if the described conditioning media response is not a "stress" response as such, the induction of GSTs by the conditioned media response may an important consideration in tumour therapy. Most malignant solid tumours are characterised by a primary tumour of relatively fast growing mass of cells. The tumour mass tends to be poorly vascularised as well as being tightly packed. The cells within the tumour may represent the *in vivo* equivalent of the highly confluent, under fed NCI H322 cells and exhibit higher levels of GST subunits through a common mechanism. The highly confluent, underfed NCI H322 cells *in vitro* may therefore represent an experimental model for the events that may occur in an *in vivo* solid tumour mass.

Immunohistochemical analysis of cultures grown to high density showed clusters of cells stained for higher levels of Alpha and Pi class GSTs. This appearance of foci showing changes in drug resistance phenotype is reminiscent of preneoplasia models of the liver. When treated with carcinogens they show the appearance of foci which show a variety of biochemical changes including changes in GST activity (Farber *et al*, 1979; Farber, 1984; Roomi *et al*, 1985). The appearance of GST expressing cells within the NCI H322 cells may be related to these foci. The biochemical changes within the preneoplastic foci may result as a response to the increased cell growth in the mitogen stimulated cells in a situation analogous to the development of heterogeneous clusters in the NCI H322 cells as a result of their increasing density in confluent cells. In the preneoplasia models these drug resistant cells are then at an advantage due to the selection pressure of the toxin and may form morphological nodules. From these nodules a few cells may continue to develop into neoplastic cells. The majority however disappear over a period of several weeks. The epigenetic appearance of a population of cells expressing a drug resistant phenotype in response to continued growth when confluent is a potential model. The inducer for the preneoplastic biochemical changes may be the induction of growth in a confluent population of cells (tumour promotion event). These cells, perhaps through the development of cell-cell interconnections, become responsive to the factor which leads to the changes in drug resistance. The NCI H322 cells may represent a model for studying the development of these changes.

CHAPTER IX

References

IX. REFERENCES

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Cell Biology

Amplification and increased expression of alpha class glutathione *S*-transferase-encoding genes associated with resistance to nitrogen mustards

(drug resistance/glutathione/preneoplasia/cancer chemotherapy)

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ABSTRACT **Glutathione-dependent enzymes play a central role in the protection of cells from cytotoxic chemicals and have been implicated in the intrinsic and acquired resistance of tumors to cytotoxic drugs. We have generated a Chinese hamster ovary line resistant to bifunctional nitrogen mustards and in this report have characterized and isolated the protein that represents the major observable phenotypic difference between the drug-sensitive and drug-resistant cell lines. This purified protein is shown to be an alpha class glutathione *S*-transferase comprising Y_cY_c subunits and possessing a pI value of ≈8.0. The intracellular level of the Y_c subunit is elevated >40-fold in the drug-resistant cell line, which could account for the increase in glutathione *S*-transferase (RX:glutathione R-transferase; EC 2.5.1.18) activity toward both 1-chloro-2,4-dinitrobenzene and cumene hydroperoxide. Other glutathione *S*-transferase subunits within this gene family are also elevated. These changes are accompanied by a significant elevation in alpha class mRNA levels. Southern analysis indicates that the genes coding for these proteins are amplified 4- to 8-fold in the drug-resistant cell line. In addition, γ-glutamyl transpeptidase [(5-glutamyl)-peptide:amino acid 5-glutamyltransferase; EC 2.3.2.2] activity is increased 3.6-fold in the drug-resistant Chinese hamster ovary cell line, which may explain the increase in cellular glutathione level. In this case no gene amplification was seen. These data indicate that gene amplification may be important in drug resistance toward alkylating agents and also that other enzymes in glutathione homeostasis are involved.**

The ability to resist chemical stresses imposed by our environment represents a major evolutionary driving force. It is clear that a wide variety of defense systems have evolved to protect against such cytotoxic insults. Studies into tumor cell drug resistance and carcinogenesis have greatly increased our understanding of these systems, and various defense mechanisms appear to be important, including membrane permeability (1), DNA repair (2), gene amplification (3), and drug detoxification (4).

For several decades glutathione (GSH) and glutathione-dependent enzymes have been implicated in drug-detoxification reactions (5). Increased glutathione content, resulting in increased drug detoxification, was proposed as a mechanism of drug resistance in tumor cells over 20 years ago (6, 7). However, only recently have these observations become a major topic of study. There is now substantial evidence indicating that the overexpression of glutathione and glutathione-dependent enzymes is an important mechanism of acquired drug resistance in both normal and tumor cells (4–13).

In a previous report we described a chlorambucil-resistant Chinese hamster ovary (CHO) cell line (CHO-Chl^r), also cross-resistant to other bifunctional nitrogen mustards (14). These cells have elevated GSH and glutathione *S*-transferase (GST; RX:glutathione R-transferase; EC 2.5.1.18) activity but do not exhibit differences in drug accumulation or DNA repair (15). The major observable phenotypic difference between the sensitive and resistant cell line is in the expression of a protein of molecular mass ≈25 kDa. Here we report the isolation and characterization of this protein, which is shown to be a GST that is overexpressed, apparently due to gene amplification. In addition, we also show that other GST subunits and γ-glutamyl transpeptidase (γGT) are elevated in the resistant line.

The cytosolic GSTs are dimeric proteins. Three structurally distinct gene families have been identified and have been termed alpha (basic), mu (neutral), and pi (acidic) (16). Within the alpha and mu groups several proteins (subunits) that can form either homo- or heterodimers and are the products of separate genes have been identified. Y_a and Y_c subunits are from the alpha family and Y_b and Y_f subunits are from the mu and pi families, respectively.

MATERIALS AND METHODS

All chemicals were purchased from commercial sources and were of the highest grade available.

Cell Culture. The two cell lines used in this study have been termed CHO-K1 (wild type) and CHO-Chl^r. The latter cell line exhibits a 24-, 34-, and 14-fold higher resistance to chlorambucil, mechlorethamine, and melphalan, respectively (14). Cells were maintained in Ham's F10 medium supplemented with 5% fetal calf (and in some cases 5% newborn calf) serum (vol/vol), streptomycin (100 μg/ml), and penicillin (100 international units/ml) and cultured in 100% humidity and 5% CO_2 as monolayers at 37°C.

Isolation of Subcellular Fractions. Cells were harvested from confluent cultures with 0.1% (wt/vol) trypsin and 0.001% (wt/vol) versene, washed three times in phosphate-buffered saline (PBS; 140 mM NaCl/2.7 mM KCl/8 mM $NaPO_4$, pH 7.4) and resuspended in 400 μl of this buffer.

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Abbreviations: CHO, Chinese hamster ovary; GST, glutathione *S*-transferase; GSH, reduced glutathione; γGT, γ-glutamyl transpeptidase; CHO-Chl^r, chlorambucil-resistant CHO cell line.

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Samples were then sonicated with three 5-sec pulses at maximal power with a 5-sec cooling period at 4°C between each step. The resulting sonicate was centrifuged at 18,000 × *g* for 20 min, and the supernatant was decanted. Protein was estimated in freshly prepared cell fractions by the method of Lowry *et al.* (17) with bovine serum albumin used as the standard. Cell fractions were stored in PBS at −70°C before further biochemical analyses were undertaken; samples were stable under the storage conditions used.

Enzymic Assays. GST activity toward the substrates 1-chloro-2,4-dinitrobenzene, ethacrynic acid, and cumene hydroperoxide were determined in the supernatant fractions by the methods of Habig *et al.* (18) and Stockman *et al.* (19). Glutathione reductase was assayed fluorometrically, as described by Weiss *et al.* (20), and glutathione peroxidase activity was determined spectrophotometrically (21). The activity of γ-glutamylcysteine synthetase, the rate-determining enzyme of GSH biosynthesis, was determined using a coupled reaction by measuring the rate of oxidation of NADH spectrophotometrically (22). The particulate cell fraction of the cell preparation was taken for the assay of γGT using γ-L-glutamyl-7-amino-4-methylcoumarin as substrate (Universal Biologicals, Cambridge) (23).

Western (Immunologic) Blot Analysis. Sodium dodecyl sulfate (SDS)/PAGE was performed according to the method of Laemmli (24). Western blots were done using essentially the method described by Towbin *et al.* (25) as modified by Adams *et al.* (26). The antibodies used were raised against the human pi class GST (λ, Y_fY_f subunits), human alpha class GST (B_1B_1, Y_aY_a subunits), and human mu class GST (μ, Y_bY_b subunits). In addition, antisera to the rat alpha class GST, Y_aY_a or Y_cY_c subunits, were also used. Antisera were prepared as described (27, 28).

Two-dimensional electrophoresis was carried out according to the method of O'Farrell (29) as modified by Robson *et al.* (14). A pH 5–8 gradient was employed in the isoelectric focusing step, which represented the first dimension. Either 100 μg of total cellular protein or 5 μg of purified protein was loaded for each experiment.

Purification of GST. Cells from the CHO-Chl^r (5×10^9 cells) were harvested, frozen at −70°C, and lysed as described by Soma *et al.* (30). Briefly, the pellets were thawed and suspended at 4°C in 40 mM Tris·HCl buffer, pH 7.4, containing 160 mM KCl, 4 mM EDTA, and 5 mM dithiothreitol. The final volume was 5 ml. The suspension was homogenized with a Teflon/glass homogenizer (25 strokes), centrifuged for 30 sec in an Eppendorf Microfuge, and then centrifuged for a further 45 min at 35,000 rpm (Kontron TST 50.13 rotor). The resulting supernatant was applied to a column of *S*-hexylglutathione (Sigma) (4-ml bed volume) equilibrated with 10 mM Tris·HCl, pH 7.8, containing 0.2 M NaCl and 3 mM 2-mercaptoethanol. The column was washed with the above buffer, and the GST was eluted by the addition of 5 mM *S*-hexylglutathione to the running buffer. With this procedure the majority of the GST in the cell lysate was retarded by the affinity gel but eluted in the wash fraction from the column. The enzyme-containing fractions were combined, dialyzed against 20 mM Tris·HCl, pH 7.8/1 mM EDTA/5 mM mercaptoethanol, and reapplied to the *S*-hexylglutathione-Sepharose column. The GST, which was now retained by the affinity matrix after this second chromatographic step, was then eluted with a solution of 5 mM *S*-hexylglutathione in the 20 mM Tris·HCl running buffer, pH 7.8. Two proteins were detected in the eluate, a minor component that eluted immediately and a major protein with GST activity. This protein was of high purity as judged by SDS/PAGE (see *Results*).

DNA and RNA Analysis. A full-length cDNA clone alpha class GST [pMP 37, 942 base pairs (bp)] was isolated from a human liver λgt11 library, which was a gift from U. Meyer (Biocenter, Basel), using an oligonucleotide coding for the six NH_2-terminal amino acids of the B_1B_1 protein. Over the coding region this cDNA had an identical sequence to that published by Board and Webb (31). A rat liver γGT cDNA clone containing the full coding sequence was isolated from a liver cDNA library in λGT10 from an ethoxyquin-treated male Fischer rat using a cDNA to the rat kidney enzyme (32). *Eco*RI digest of this clone yielded two fragments. The 5′ fragment of 1230 bp (pEGL1.1) was used as a probe.

DNA Isolation. DNA isolation was done essentially as described by Maniatis *et al.* (33). Cells (10^7) were washed twice with PBS and then harvested by scraping into PBS. Cells were then lysed in 0.5% SDS/150 mM NaCl/10 mM EDTA/10 mM Tris·HCl, pH 7.5 (2 ml) and treated with RNase A (100 μg/ml) and then proteinase K (250 μg/ml) at 37°C for 1 hr and 4 hr, respectively. Samples were extracted with phenol followed by phenol/chloroform (1:1 vol/vol), and the DNA was precipitated in 60% EtOH containing 1 M ammonium acetate. The precipitated DNA was wound onto a glass rod, air dried, and resuspended in 10 mM Tris·HCl, pH 7.5/1 mM EDTA. RNA was isolated using the method described by Birnboim (34). DNA and RNA concentrations were determined from the absorption at 260 nm. Southern and Northern (RNA) analysis were done as described by Hill *et al.* (35).

RESULTS AND DISCUSSION

The levels of a variety of glutathione-dependent enzymes in the drug-sensitive and resistant CHO cell lines are shown in Table 1. In agreement with a previous report (15), both the GSH level and the GST activity (toward 1-chloro-2,4-dinitrobenzene) were significantly higher in the chlorambucil-resistant cell line (1.8- and 2.7-fold, respectively). In addition, the peroxidase activity toward cumene hydroperoxide was also elevated 5.1-fold. This increase appeared to be completely due to differences in GST expression, as the selenium-dependent activity (determined using H_2O_2 as substrate) was similar in both cell lines (Table 1). The majority of the peroxidase activity toward cumene hydroperoxide in the CHO-K1 cells was mediated by the selenium-dependent enzyme. When this contribution was subtracted from the values obtained, CHO-Chl^r had ≈50-fold higher GST-mediated peroxidase activity than the wild-type cell line.

A variety of enzymes are involved in maintaining the levels of reduced glutathione within cells (36). Three of these—

Table 1. Glutathione-dependent enzyme expression in CHO-K1 and CHO-Chl^r cell lines

	Enzyme activity							
		GST						
Cell line	GSH	CDNB	EA	CHP	GPX, H_2O_2	γGT	γGCS	GRD
CHO-K1	18.4 ± 2.2	239 ± 36	19.7 ± 3.2	16.4 ± 2.0	8.9 ± 4.8	0.82 ± 0.26	70.0 ± 16.2	96.5 ± 23
CHO-Chl^r	33.0 ± 5.6	638 ± 115†	38.0 ± 12.1‡	83.4 ± 18.3*	10.0 ± 2.7	2.85 ± 0.48*	97.7 ± 51.8	129.5 ± 61.5

Values are expressed as nmol per min/mg of protein for 1-chloro-2,4-dinitrobenzene (CDNB), cumene hydroperoxide (CHP), ethacrynic acid (EA), H_2O_2, γ-glutamylcysteine, synthetase (γGCS), and glutathione reductase (GRD) or nmol per min/10^6 cells for γGT. GSH is expressed as nmol/mg of protein. GPX, glutathione peroxidase. *$P < 0.005$, $^\dagger P < 0.01$, $^\ddagger P < 0.07$.

glutathione reductase, γGT, and γ-glutamylcysteine synthetase—were measured. Only γGT was elevated significantly in the drug-resistant cell line, although some change in γ-glutamylcysteine synthetase and glutathione reductase was observed. This suggests that these proteins, in particular γGT, may be responsible for maintaining the elevated cellular GSH level. Elevated γGT concentrations have been seen in cell lines resistant to cytotoxic drugs (37, 38). In addition, the fact that preneoplastic foci in rat liver, which have elevated GSH content, also have elevated γGT levels (39) supports the hypothesis that this enzyme is involved in maintaining increased cellular GSH levels. It is interesting that in the models for drug resistance induced by alkylating agents that we have studied, γGT activity is consistently elevated (ref. 38, and C.R.W. and A.D.L. unpublished data).

Our initial studies indicated that altered expression of the alpha class GST represented the major change in GST expression in the CHO-Chl^r cell line (15). In support of this, the staining pattern of cytosolic samples clearly showed an elevation in a protein with a mobility similar to that of the rat Y_c GST subunit (Fig. 1 arrow). This subunit could, therefore, be the polypeptide with an estimated molecular size of 25 kDa, which was previously shown to be dramatically overexpressed in the CHO-Chl^r cell line (15); more recent work has estimated the molecular size of the Y_c subunit to be 27–27.5 kDa (40).

To establish whether this was the case, the overexpressed protein was purified. In addition, Western blots of CHO cytosol with antibodies to known rat or human GST subunits (Y_a, Y_b, Y_c, and Y_f) were done. A two-step purification procedure (Fig. 2) yielded a pure protein of similar mobility to the rat Y_c (molecular mass, 27.5 kDa) standard. This protein was shown to be a GST based on its activity towards 1-chloro-2,4-dinitrobenzene, cumene hydroperoxide, and ethacrynic acid, the turnover numbers for these substrates being 6031, 964, and 550 nmol per min/mg of protein, respectively. The relative activities of the Chinese hamster GST toward these three substrates are similar to that exhibited by the rat Y_c subunit (41). Western blots showed that the purified protein and the protein overexpressed in CHO-Chl^r reacted strongly with antibodies to the alpha class rat Y_c GST subunit (Fig. 3). This blot also demonstrated a 40-fold difference in the expression of this protein between the drug-sensitive and resistant cell lines. This result would agree with the 50-fold elevation in GST-mediated peroxidase activity. To confirm these findings the mobility of the purified protein after electrophoresis in two-dimensional polyacrylamide gels was compared with the overexpressed protein in the CHO-Chl^r cells after two-dimensional electrophoresis. The two proteins were found to have identical mobilities in this system (Fig. 4). The isoelectric point of the purified protein was ≈pH 8.0.

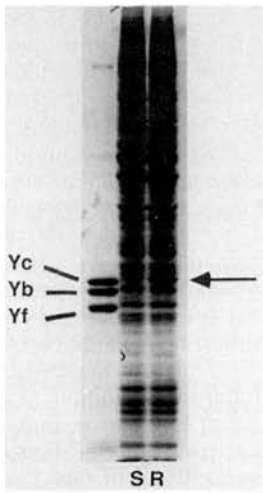

FIG. 1. Protein staining pattern of CHO-K1 and CHO-Chl^r. Samples were separated by SDS/PAGE. Fifty micrograms of soluble protein was taken per lane. Mobilities of the rat Y_c, Y_b, and Y_f standards are also shown. CHO-K1 and CHO-Chl^r are the wild type (S) and resistant (R) cell lines, respectively.

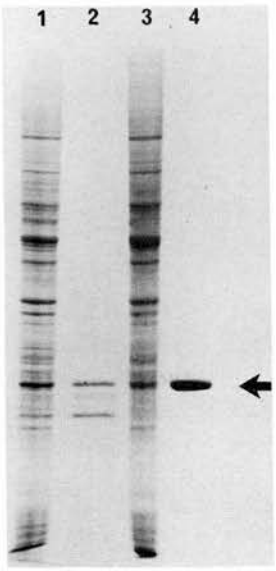

FIG. 2. Purification of the overproduced protein of molecular mass 27.5 kDa from CHO-Chl^r cells. Samples were taken at various stages during the purification and run on 11% SDS/PAGE gels and stained with Coomassie blue. Lanes 1, 2, 3, and 4 are total cell extract, peak of GST activity eluted from the *S*-hexylglutathione column, column flowthrough in the presence of 0.2 M salt, and the purified GST obtained after rechromatography of sample in lane 3, respectively.

In view of the observation that other GST subunits may be involved in drug resistance, Western blots were done with antibodies to other GST enzymes (Fig. 5). Slight differences between CHO-K1 and CHO-Chl^r in the expression of the pi and mu class proteins were seen. The pi class GST is present at much higher concentrations than the alpha class proteins in the CHO-K1 CHO cells and will account for most of the 1-chloro-2,4-dinitrobenzene activity in these cells. This subunit does not have activity toward cumene hydroperoxide, which will explain the much smaller difference in 1-chloro-2,4-dinitrobenzene relative to peroxidase activity seen between CHO-K1 and CHO-Chl^r. A significant elevation in the Y_a subunit, another alpha class protein, was seen. The antibody to this subunit also cross-reacted weakly with the CHO Y_c subunit (Fig. 5). The change in the expression of alpha class GST protein was accompanied by a much higher level of the mRNA coding for these proteins (Fig. 6).

To establish the molecular mechanism for the overexpression of alpha class GST, DNA from the cell lines was analyzed by Southern blot analysis (Fig. 7). The complexity of the banding pattern obtained together with the identification of two alpha class proteins (Y_a and Y_c) indicates that this is a multigene family; this result is consistent with the findings in other species. In Chinese hamsters this gene family appears to cover up to 100 kb of DNA. The intensity of almost all bands was much higher for CHO-Chl^r cells, and in view of the specificity of the cDNA probe for the alpha class GST gene family, the amplified bands almost certainly contain the genes for the Y_a and Y_c proteins. Based on limited dilution of CHO-Chl^r DNA, until the bands were of equal intensity to CHO-K1, it appeared that there was a 4- to 8-fold elevation in gene copy number (data not shown). Densitometric scan-

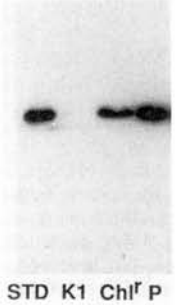

FIG. 3. Comparison of GST subunit content in CHO-K1 and CHO-Chl^r and the purified protein. Western blots were carried out as described. Y_c, standard; K1 and Chl^r, samples from the sensitive and resistant cell lines, respectively; P, purified GST protein (5 μg). The antibody used was to the rat Y_c subunit.

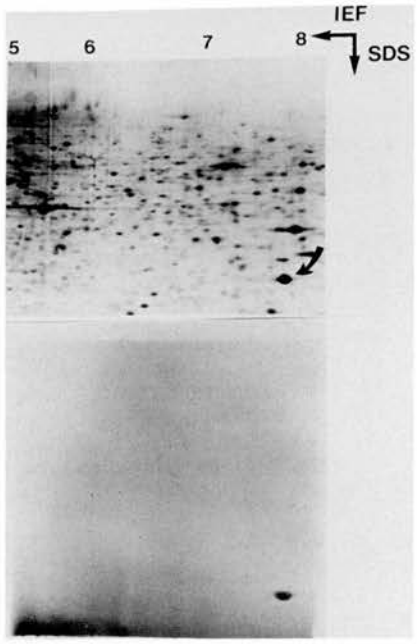

FIG. 4. Two-dimensional gel electrophoresis of CHO-Chl^r and the purified GST protein. Gels were run as described in the text and in ref. 14. One hundred micrograms of soluble protein from CHO-Chl^r (*Top*) or 5 μg of the purified GST enzyme (*Bottom*) were taken. The overexpressed protein in CHO-Chl^r is indicated by a curved arrow. IEF, isoelectric focusing.

ning of the bands indicated that the increase may be up to 20-fold. No difference between CHO-K1 and CHO-Chl^r was seen when a γGT cDNA was used as a probe (Fig. 7*B*), indicating that the change in γGT activity was not due to an amplification event.

The above data provide strong evidence that the class alpha Y_a and Y_c GST are the major proteins overexpressed in CHO cells made resistant to chlorambucil. This increase in expression appears to be a consequence of increased transcription and mRNA levels resulting from a gene amplification. A direct relationship between these effects, however, requires further study. It is worthy of note that DNA repair capacity and also drug accumulation are unchanged in the CHO-Chl^r cells (15) and that the mechanism of the drug resistance appears to be due to enhanced drug detoxification, which results in reduced DNA damage (15). The alpha class GST subunits, therefore, appear important in this mechanism. In support of this possibility GSTs have been shown to catalyze the conjugation of melphalan to GSH, leading to its detoxification (42). Melphalan is a structural homologue of chlorambucil, to which CHO-Chl^r is also resistant. Which GST subunit is responsible for this reaction, however, has not been established.

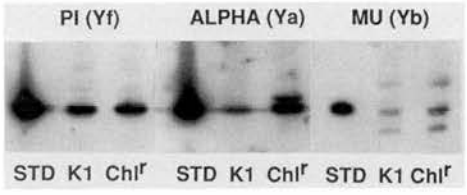

FIG. 5. GSH subunit content in CHO-K1 and CHO-Chl^r. Western blots were done as described with 50 μg of soluble protein per track. The bands were identified by the antibodies raised to the human Y_f, class PI; Y_a, class ALPHA; and Y_b, class MU GST subunits; STD, human GST standard (1 μg); K1, CHO-K1; Chl^r, CHO-Chl^r.

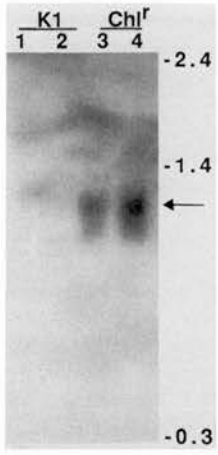

FIG. 6. Alpha class GST mRNA levels in CHO-K1 and CHO-Chl^r. Twenty micrograms of RNA from separate cultures (lanes: 1 and 2, CHO-K1; 3 and 4, CHO-Chl^r) was separated on 1.5% agarose gels containing 6% (wt/vol) formaldehyde and then transferred to Hybond-N membrane. Subsequent analysis was carried out according to Hill *et al.* (35). Molecular sizes in kilobases (kb) are given at right. Arrow, alpha GST.

The molecular mechanisms responsible for the changes in the levels of GST and other glutathione-dependent enzymes have remained elusive. However, we have now obtained evidence that gene amplification may be an important part of this process and, therefore, may play an important role in drug resistance induced by alkylating agents. The time point at which this amplification occurs—i.e., whether some cells

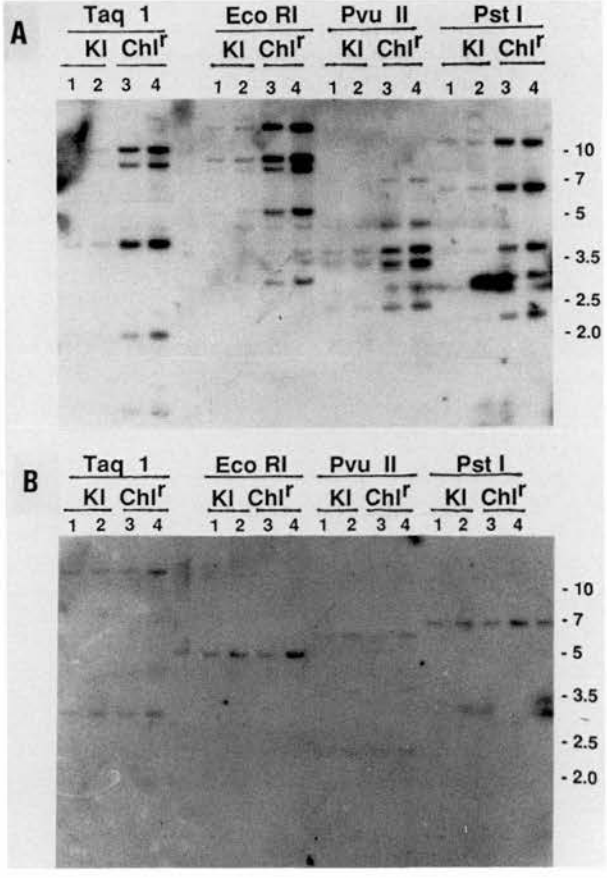

FIG. 7. DNA analysis of CHO-K1 and CHO-Chl^r using an alpha class GST and γGT cDNAs as probes. Two DNA samples from separate cultures for each cell line were digested with restriction endonucleases according to the manufacturer's instructions. (Lanes: 1 and 2, CHO-K1 (K1); 3 and 4, CHO-Chl^r). DNA (5 μg) was separated on a 1% agarose gel and transferred to Hybond-N. Fragments hybridizing the human alpha class GST cDNA (*A*) or the γGT cDNA (*B*) were detected as described by Hill *et al.* (35). On the basis of absorption at 260 nm 1.4-fold more DNA was loaded in lane 4. Molecular sizes in kb are given at right.

intrinsically contain amplified sequences or whether gene amplification is induced by chemical exposure—is unclear.

The phenotypic changes observed in protein expression in drug resistance—i.e., in glutathione-dependent enzymes, P-glycoprotein, and DNA repair enzymes—bear a remarkable similarity to those seen in chemical-induced neoplasia in rat liver (12, 43–45). These changes are often independent of the chemical reagent used and may well reflect the existence of a fundamental cellular response mechanism to combat chemical insult. Whether common genetic changes are involved in such a response is at present unknown.

The role of glutathione-dependent enzymes in this adaptive change is uncertain. However, the ubiquitous nature of the changes seen in the expression of these proteins after cytotoxic insult indicates that they are important. For example, changes in levels of these enzymes have been reported to occur during oxidative stress in both bacteria (46) and rodent lung (47) and also in chemical-induced stress in the mouse bone marrow (13, 48). With particular regard to the GST, it is known that in preneoplasia in rat liver the Y_a, Y_b, Y_c, and Y_f GST subunits are overexpressed (12, 43, 44, 49, 50). The Y_f subunit has also been shown to be overexpressed in MCF7 cell lines made resistant to doxorubicin (adriamycin) (51) or to a variety of other chemotherapeutic agents (Hill B.J., Karen, P. and C.R.W, unpublished). Our study shows that in tumor cell drug resistance the expression of other GST subunits, as well as a variety of glutathione-dependent enzymes, could be of significance in the resistance mechanism. Overexpression of these genes, for example, as a consequence of a gene amplification, implies the presence of another form of cross-resistance distinct from the multidrug resistance phenotype that involves alkylating agents and compounds that are themselves, or which generate, peroxides.

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